The nuclear receptor gene *nhr-25* plays multiple roles in the *C. elegans* heterochronic gene network to control the larva-to-adult transition

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Abstract

Developmental timing in the nematode *Caenorhabditis elegans* is controlled by heterochronic genes, mutations in which cause changes in the relative timing of developmental events. One of the heterochronic genes, *let-7*, encodes a microRNA that is highly evolutionarily conserved, suggesting that similar genetic pathways control developmental timing across phyla. Here we report that the nuclear receptor *nhr-25*, which belongs to the evolutionarily conserved *fushi tarazu-factor 1*/nuclear receptor NR5A subfamily, interacts with heterochronic genes that regulate the larva-to-adult transition in *C. elegans*. We identified *nhr-25* as a regulator of *apl-1*, a homolog of the Alzheimer's *amyloid precursor protein-like* gene that is downstream of *let-7* family microRNAs. NHR-25 controls not only *apl-1* expression but also regulates *let-7*-dependent developmental timing. NHR-25 negatively regulates the expression of the adult-specific collagen gene *col-19* in lateral epidermal seam cells. In contrast, NHR-25 positively regulates the larva-to-adult transition for other timed events in seam cells, such as cell fusion, cell division and alae formation. The genetic relationships between *nhr-25* and other heterochronic genes are strikingly varied among several adult developmental events. We propose that *nhr-25* has multiple roles in both promoting and inhibiting the *C. elegans* heterochronic gene pathway controlling adult differentiation programs.

Keywords: *Caenorhabditis elegans*, heterochronic gene, developmental timing, *let-7*, nuclear receptor; *nhr-25*

Introduction

The temporal coordination of cell proliferation and differentiation during development is essential for the correct morphogenesis of multicellular organisms (Banerjee and Slack, 2002). The development of the nematode *Caenorhabditis elegans* serves as an excellent model to study how the temporal fates of cells are specified (Ambros, 2000). *C. elegans* development progresses through six stages: the embryonic stage, four larval stages (L1, L2, L3 and L4) that each end with a molt and the adult stage. At each stage, the timing of development is regulated such that specific cell types are produced during a particular developmental period (Sulston and Horvitz, 1977).

Genetic analyses have revealed a number of genes, the so-called heterochronic genes, which control the temporal regulation of postembryonic development in *C. elegans* (Ambros and Horvitz, 1984; Moss, 2007; Rougvie, 2005). Mutations in these heterochronic genes cause cells to adopt fates normally expressed at earlier or later stages of development in *C. elegans*. Progression through the first and second larval stages, L1 and L2, is controlled by the microRNA (miRNA) *lin-4,* which down-regulates the activity of transcription factor LIN-14 (Ambros, 2000). Later in larval development, members of the *let-7* miRNA family control the L2-to-L3 and L4-to-adult transitions (Abbott et al., 2005; Esquela-Kerscher et al., 2005; Li et al., 2005; Reinhart et al., 2000) through the down-regulation of other heterochronic genes such as *hbl-1* (Abrahante et al., 2003; Lin et al., 2003), *lin-41* (Slack et al., 2000) and *daf-12* (Grosshans et al., 2005). Recently, negative feedback networks between the *let-7* family of miRNAs and their targets have been revealed, as *daf-12* and *hbl-1* regulate *let-7* expression (Bethke et al., 2009; Hammell et al., 2009; Roush and Slack, 2009). The larva-to-adult (L/A) transition is controlled by the repression of *hbl-1* and *lin-41* through *let-7*, which in turn allows for the activation of *lin-29*. *lin-29* encodes a

transcription factor that ceases the larval developmental program and simultaneously induces adult cell differentiation (Rougvie and Ambros, 1995). Many of these heterochronic genes identified from *C. elegans* are evolutionarily conserved in genomes of diverse animal species (Moss, 2007). Some of the orthologs are conserved in terms of the regulation of developmental timing, as exemplified by the recent finding that a human ortholog of the *C. elegans* heterochronic gene *lin-28* is genetically linked with the timing of human puberty (Ong et al., 2009). Among the conserved heterochronic genes, the *let-7* miRNA family is an essential genetic component for developmental timing of various animal species, as its sequence and temporal expression pattern as well as several *let-7* target genes are highly conserved during animal evolution (Johnson et al., 2005; Pasquinelli et al., 2000).

Although a number of key heterochronic genes downstream of *let-7*, including transcription factors and signal transduction molecules, have been identified (Grosshans et al., 2005; Lall et al., 2006), the molecular mechanisms underlying the developmental timing for adult differentiation programs are less well studied. For example, although *lin-29* acts downstream of *let-7* and its targets (Bettinger et al., 1996; Rougvie, 2005), little is known about mechanisms of *lin-29* activation that promote adult differentiation. Therefore, uncharacterized genes could be acting downstream of *let-7* in the heterochronic gene pathway to specify the appropriate execution of the adult differentiation program.

Here, we report that the nuclear receptor (NR) gene *nhr-25*, which belongs to the evolutionarily conserved *fushi tarazu-factor 1 (FTZ-F1)*/nuclear receptor NR5A subfamily, regulates the L/A transition in *C. elegans*. *nhr-25* encodes one of the hugely expanded NRs found in the worm genome and orthologous to mammalian *steroidogenic factor 1* (*SF-1*) and *liver receptor homolog 1* (*LRH-1*) (Asahina et al., 2000;

Gissendanner and Sluder, 2000). Previous studies have shown that *nhr-25* is required for a wide variety of developmental events, such as embryogenesis, differentiation of larval epidermis, production of exoskeleton, vulval morphogenesis, and distal tip cell/anchor cell formation (Asahina et al., 2000; Asahina et al., 2006; Chen et al., 2004; Gissendanner and Sluder, 2000; Hajduskova et al., 2009; Silhankova et al., 2005). *nhr-25* is also known as a downstream target of *let-7* and other heterochronic genes involved in the cessation of molting (Hayes et al., 2006). Strikingly, we reveal that the phenotypes of loss of *nhr-25* function animals are not easily classified into either the "precocious" or "retarded" class of heterochronic genes. We propose that *nhr-25* plays critical roles in the heterochronic gene pathway with the unique ability to both promote and inhibit certain adult differentiation programs in *C. elegans* and that these dual roles are dictated by its ability to genetically interact with multiple heterochronic genes.

Materials and methods

Nematode strains and culture

C. elegans strains were grown at 20 ºC under standard conditions. Bristol N2 was used as the wild type. The mutant strains used were as follows:

- *hbl-1(ve18)*, a hypomorphic allele that deletes 5 base pairs nucleotides at a boundary of the second exon and the second intron of *hbl-1* (Abrahante et al., 2003).

- *hbl-1(mg285)*, a hypomorphic allele that deletes 301 base pairs in the 5´ region of *hbl-1*, causing a frameshift predicted to truncate HBL-1 protein in the third exon (Lin et al., 2003).

- *let-7(n2853)*, a temperature-sensitive, point-mutated allele that exhibits a strong retarded phenotype (Reinhart et al., 2000).

- *lin-29(n546)*, a point mutation introducing a premature opal stop codon that eliminates the C-terminal 1/3 of the protein (Ambros and Horvitz, 1984; Rougvie and Ambros, 1995).

- *lin-41(ma104)*, a hypomorphic allele that has a transposon insertion in the twelfth exon (Slack et al., 2000).

- *lin-42(n1089)*, which contains a large deletion that removes part of exon 2 and exons 3–5, disrupting wild-type activity of three out of four *lin-42* isoforms (Tennessen et al., 2006).

- *nhr-25(ku217)*, a hypomorphic allele that has a missense mutation causing L32F substitution in the DNA binding domain of NHR-25 (Chen et al., 2004).

To visualize the nuclei of seam cells, we utilized an integrated construct *wIs51[scm::gfp]*. To visualize both nuclei and junctions of seam cells, another integrated strain *wIs79[ajm-1::gfp;scm::gfp]* was used. *maIs105[col-19::gfp]* (Abbott et al., 2005) was kindly provided by C. Hammell and V. Ambros. Other transgenic strains used were *nwrIs001[apl-1::gfp::unc-54]* (Niwa et al., 2008), *ctIs37[hbl-1::gfp::unc-54]* (Lin et al., 2003), *jmEx33[nhr-25::gfp::unc-54]* (Silhankova et al., 2005) and *TP12:kaIs12[col-19::gfp]* (Thein et al., 2003).

RNAi experiments

Gene knockdown was achieved through RNAi by feeding as described (Timmons and Fire, 1998). A subset of Dr. Julie Ahringer's RNAi library (Fraser et al., 2000) that includes clones for 387 predicted *C. elegans* transcription factors (Table S1) was purchased from Geneservice. Synchronized populations of L1 larvae were fed bacteria expressing dsRNA corresponding to the target genes. In mock RNAi experiments, bacteria carrying an empty vector pPD129.36 (Kamath et al., 2003) were used. The

following PCR products used for RNAi experiments in this study were previously described (Kamath et al., 2003): sjj_F11A1.3 for *daf-12*, sjj_Y17G7A.2 for *lin-29*, sjj_C01H6.5 for *nhr-23* and sjj_F11C1.6 for *nhr-25*. We confirmed that approximately 35 % of *nhr-23(RNAi)* animals (n=73) exhibited a defect in larval cuticle shedding at the L4 molt as previously reported (Kostrouchova et al., 1998). We also observed that *daf-12(RNAi)* significantly suppressed the bursting phenotype of *let-7(n2853)* (90 %; n=126), as previously described (Grosshans et al., 2005).

Observation of worms

Except the *lin-42(n1089)* worms, L4 animals were staged by the relative positions of their gonadal distal tip cells to the vulva: early, mid and late L4 animals were defined as animals showing 0-1/4, 1/4-1/2 and >1/2 gonadal turns, respectively, as previously described (Niwa et al., 2008). *lin-42(n1089)* L4 worms were staged by degrees of vulval invagination, because *lin-42(n1089)* exhibits precocious turning of gonadal tips as previously described (Tennessen et al., 2006). To observe GFP signals in seam cells of the *apl-1::gfp* transgenic lines, we observed all seam cells except the few cells surrounding the head and pharyngeal regions; the non-seam expression of *apl-1::gfp* in these regions is strong (Niwa et al., 2008) and interferes with evaluation of the GFP expression in seam cells. Therefore, "GFP in all seam cells", as described in Fig. 1, means the *apl-1::gfp* animals showing GFP expression in all seam cells except the cells in the head region. A scanning election microscopic observation was performed as previously described (Silhankova et al., 2005) except worms were fixed in 2.5% glutaraldehyde in PBS buffer and images were captured with JEOL JSM-7401F.

Results

nhr-25 is required for *apl-1* expression in seam cells

Our previous work had demonstrated that the expression of *apl-1* in seam cells is tightly regulated and repressed by the *let-7* family miRNAs after the L4 stage (Niwa et al., 2008). This repression is controlled by heterochronic genes that act downstream of *let-7*, such as *hbl-1*, *lin-41*, and *lin-42*, but not *lin-29*. The expression of *apl-1* in seam cells is strongly induced only at the L4/A transition, as no or weak *apl-1* expression is detected in seam cells from L1 to early L4 stages (Fig. S1) (Niwa et al., 2008). Therefore, we utilized *apl-1* expression as a new approach to elucidate *let-7*-dependent terminal differentiation pathways at the L/A transition in *C. elegans* (Niwa et al., 2008). We performed an RNAi screen to search for genes that affected *apl-1::gfp* expression in an integrated transgenic line carrying the full length *apl-1* promoter region (-6997 to -1 bp) upstream of a *gfp* construct (Niwa et al., 2008). We targeted 387 predicted *C. elegans* transcription factors (Table S1) using a subset of commercially available Ahringer's RNAi library (Fraser et al., 2000). In the RNAi screen, we found that *apl-1::gfp* expression in seam cells in the L4 stage was greatly reduced by RNAi against the *nhr-25* gene, which encodes an evolutionarily conserved NR from cnidarians to human (Asahina et al., 2000; Escriva et al., 1997; Gissendanner and Sluder, 2000) (Figs. 1A, B and S2A, B). While *apl-1::gfp* expression in seam cells in the L4 stage was also reduced by RNAi against three additional transcription factor genes, *nhr-195*, *elt-2* and *lin-11* (Table S1), we did not observe any other heterochronic phenotypes, including changes in timing of alae formation and adult-specific collagen gene expression, by RNAi against any of these three genes (data not shown). Therefore, we focused on the *nhr-25* gene for detailed analysis as described below.

The *apl-1* reduction phenotype described above was specific to *nhr-25(RNAi)* and was not an off-target effect because *apl-1* expression in seam cells in the late L4 stage was also diminished in the *nhr-25(ku217)* mutant (Fig. 1D), a hypomorphic recessive allele of *nhr-25* that causes an amino acid substitution in the DNA binding domain of NHR-25 (Chen et al., 2004). *nhr-25(RNAi)* and *nhr-25(ku217)* did not affect *apl-1* expression in pharyngeal cells, in which constant *apl-1* expression was observed throughout development in wild-type worms (Fig. S3) (Niwa et al., 2008). While a slight decrease of the *apl-1* expression level was observed in uterine pi cells in the *nhr-25(RNAi)* worms at the late L4 stage as compared to wild type (Fig. S3), a dramatic reduction of *apl-1* expression was only detected in seam cells.

The genome of *C. elegans* is predicted to encode 284 NRs (Gissendanner et al., 2004; Magner and Antebi, 2008). Besides *nhr-25*, the *daf-12* and *nhr-23* NRs have also been previously shown to play a crucial role in differentiation and gene expression in seam cells (Brooks et al., 2003; Hayes et al., 2006; Kostrouchova et al., 1998; Kostrouchova et al., 2001). Therefore, we examined whether the loss of *nhr-23* or *daf-12* function influenced *apl-1* expression in seam cells during L4. RNAi against mock, *nhr-23* or *daf-12* showed no obvious changes in temporal *apl-1::gfp* expression in seam cells (Figs. 1A, C, D and S4), while *nhr-23(RNAi)* resulted in molting defects and *daf-12(RNAi)* suppressed the vulval bursting phenotype of the *let-7* mutant as previously reported (Grosshans et al., 2005; Kostrouchova et al., 1998) (See Materials and Methods). These results show that NHR-25 is the major transcriptional regulator that positively controls *apl-1* expression in the late L4 stage.

nhr-25 and *apl-1* are synergistically required for molting

apl-1 is involved in the molting process in *C. elegans* (Hornsten et al., 2007; Niwa et al.,

2008). Because *nhr-25* is also required for *C. elegans* molting (Asahina et al., 2000; Gissendanner and Sluder, 2000; Hayes et al., 2006), we examined whether *nhr-25* genetically interacts with *apl-1* to regulate molting. *nhr-25(ku217); apl-1(RNAi)* double mutants showed a growth defect during the L4 stage and typically could not shed their larval cuticle at the L4 molt (Fig. 2A, B), whereas *nhr-25(ku217)* or *apl-1(RNAi)* alone only rarely showed the phenotype (Fig. 2B). This genetic interaction was similar to the previously reported interaction between of *apl-1* with *hbl-1*, *lin-41* and *lin-42* (Niwa et al., 2008). We have also examined a possible genetic interaction of *apl-1* and *nhr-25* with *lin-29* on the molting defect at L4 molt, but no obvious interactions were detected (Fig. S5).

It is known that *nhr-25* and *apl-1* are both required for shedding larval cuticles throughout *C. elegans* larval development (Gissendanner and Sluder, 2000; Hornsten et al., 2007). However, NHR-25 and APL-1 seem to be required predominantly at L4 to the adult transition, most likely *apl-1* acting downstream of *nhr-25*, as the robust expression of *apl-1* in seam cells at the late L4 stage is regulated by NHR-25 (Fig. 1A, B, D) and their loss of functions mutually enhance the L4 molting defect.

nhr-25 synergistically acts with _hbl-1_, _lin-41_ and _lin-42_ to negatively regulate the expression of adult-specific collagen gene _col-19_

nhr-25 appears to be directly targeted by the *let-7* miRNA and its family member *mir-84*, and *let-7*-dependent repression of *nhr-25* in the adult stage is essential for inhibiting the molting process characteristic of larvae (Hayes et al., 2006). Meanwhile, *hbl-1*, *lin-41* and *lin-42*, the heterochronic genes essential for adult differentiation in seam cells, are also downstream of *let-7* (Abrahante et al., 2003; Banerjee et al., 2005; Lin et al., 2003; Slack et al., 2000). Our previous study revealed that *hbl-1*, *lin-41* and *lin-42* positively

regulate *apl-1* expression in seam cells in the late L4 stage (Niwa et al., 2008). Therefore, the positive regulation of *apl-1* expression by *nhr-25* in seam cells described above prompted us to investigate whether loss of *nhr-25* function caused heterochronic phenotypes in seam cells. We first examined the hypodermal expression pattern of *col-19*, encoding an adult-specific collagen protein. *col-19* is known to be a direct target of the heterochronic transcription factor LIN-29 (Abrahante et al., 1998; Rougvie and Ambros, 1995). In wild type or control (mock) RNAi animals, *col-19* expression begins at mid-late L4 stage at low level (Figs. 3A, F and S6). In contrast, similar to the depletion of *hbl-1*, *lin-41* or *lin-42* (Abrahante et al., 2003; Jeon et al., 1999) (data not shown), *nhr-25(RNAi)* or the *nhr-25(ku217)* mutation triggered strong precocious *col-19::gfp* expression in the mid-L4 stages (Figs. 3B, F, and S7A). *nhr-23(RNAi)* did not induce such precocious *col-19* expression (Figs. 3F and S7B). This precocious induction of *col-19::gfp* by *nhr-25(RNAi)* was not suppressed by the *lin-29* loss-of-function mutation (Fig. 3F), suggesting that *nhr-25* regulates *col-19* expression independently from *lin-29*.

We further examined whether *nhr-25* showed a genetic interaction with *hbl-1*, *lin-41* and *lin-42* to regulate *col-19* expression. Single depletion of *nhr-25*, *hbl-1*, *lin-41*, or *lin-42* induced a high level of the precocious *col-19* expression after the mid L4 stage (Figs. 3B, F and S7A) but only a subtle level of the precocious *col-19* expression in the early L4 stage (Fig. 3C, D, G) (Niwa et al., 2008). In contrast, a combination of *nhr-25* RNAi with either an *hbl-1*, *lin-41*, or *lin-42* loss-of-function mutation dramatically increased the precocious *col-19::gfp* expression in seam cells in the early L4 stage (Fig. 3E, G). These results suggest that *nhr-25* synergistically acts with *hbl-1*, *lin-41*, and *lin-42* to negatively regulate *col-19* expression.

nhr-25 is required for precocious *apl-1* expression in seam cells in *hbl-1*, *lin-41* and *lin-42* mutants

Although *hbl-1*, *lin-41* and *lin-42* positively regulate *apl-1* expression in the late L4 stage as we have reported previously (Niwa et al., 2008), these genes also play a role in the negative regulation of *apl-1* expression in the early L4 stage or earlier. *apl-1* expression starts from the mid L4 stage in wild-type worms, and loss of function of *hbl-1*, *lin-41* or *lin-42* causes precocious *apl-1* expression in the early L4 stage (Figs. 1E, H and S2C) (Niwa et al., 2008). We examined whether the precocious *apl-1* expression in the early L4 stage induced by these heterochronic mutations required *nhr-25* activity. As compared to mock RNAi (Figs. 1E and S2C), *nhr-25(RNAi)* greatly suppressed the precocious *apl-1* expression in seam cells in *hbl-1(mg285)* (Figs. 1F, H and S2D), *lin-42(n1089)* (Fig. 1H; data not shown) and *lin-41(ma104)* mutants (data not shown). This suppression was specific to *nhr-25(RNAi)*, as the negative control *nhr-23(RNAi)* did not induce any change in *apl-1* expression in seam cells (Fig. 1G, H). These results suggest that *nhr-25* regulates *apl-1* expression downstream of *hbl-1*, *lin-41* and *lin-42*, and also indicate that *nhr-25* counteracts *hbl-1*, *lin-41* and *lin-42* to control *apl-1* expression in the early L4 stage. The action of *nhr-25* in early L4 is opposite and paradoxical to the fact that *nhr-25*, *hbl-1*, *lin-41* and *lin-42* cooperate to positively induce *apl-1* expression in the late L4 stage.

nhr-25 antagonizes the functions of *hbl-1*, *lin-41*, and *lin-42* to positively regulate adult alae formation and seam cell fusion

The previous data imply that the genetic interaction of *nhr-25* and *hbl-1*, *lin-41* and *lin-42* is not uniform. The inconsistency of these genetic relationships prompted us to examine whether and how *nhr-25* regulates adult differentiation programs apart from

col-19 expression. First, we examined whether *nhr-25* is involved in the temporal control of adult alae formation, which is an adult-specific cuticular structure secreted from seam cells (Ambros and Horvitz, 1984). Whereas loss of *nhr-25* function has been previously reported to cause abnormal adult alae morphology (Silhankova et al., 2005), genetic interactions between *nhr-25* and heterochronic genes have not been elucidated. Adult alae formation is temporally regulated by *hbl-1*, *lin-41* and *lin-42*, as loss-of-function mutations of these genes cause precocious alae formation in the early L4 stage (Fig. 4A, B, D) (Abrahante et al., 2003; Abrahante et al., 1998; Jeon et al., 1999; Lin et al., 2003; Slack et al., 2000). Our above-described result about the precocious *col-19* expression implied that the precocious alae formation in the absence of either *hbl-1*, *lin-41* or *lin-42* function should also be enhanced by *nhr-25(RNAi)*. However, *nhr-25(RNAi)* significantly suppressed the precocious alae formation in these mutant backgrounds (Fig. 4C, D), and no precocious alae defect was observed in *nhr-25(RNAi)* alone (Fig. 4D). It is noteworthy that the penetrance of precocious alae phenotypes was much higher than that of precocious *col-19* expression (Fig. 3G) in the early L4 stage of *hbl-1*, *lin-41* and *lin-42* mutants. These results suggest that the timing of expression of precocious phenotypes are independent between alae formation and *col-19* expression in seam cells, or reflect a delay in GFP accumulation.

We also examined whether the activity of *nhr-25* modulated the timing of seam cell fusion. In wild type animals, seam cells divide with a stem cell-like pattern during larval stages before exiting the cell cycle, and these cells terminally differentiate and fuse each other around the time of the L4 molt (Sulston and Horvitz, 1977). In addition to abnormal adult alae formation, loss-of-function mutations of *hbl-1* and *lin-42*, precociously induce the seam cell fusion even in the early L4 stage (Fig. 5A, C) as compared to wild-type worms (Figs. 5C and S8A) (Abrahante et al., 2003; Banerjee et

al., 2005; Lin et al., 2003). In contrast, *nhr-25(RNAi)* decreased the numbers of individual worms exhibiting precociously fused seam cells in *hbl-1* and *lin-42* mutants in the early L4 stage (Fig. 5B, C) but did so less efficiently than mutations of the master adult switch gene *lin-29* (Abrahante et al., 2003; Abrahante et al., 1998; Jeon et al., 1999; Lin et al., 2003). *nhr-25(RNAi)* itself also prevented seam cell fusion in the late L4 stage (Figs. 5C and S8B). These results suggest that *nhr-25* is negatively regulated by and acts antagonistically to *hbl-1*, *lin-41* and *lin-42* during seam cell fusion as well as adult alae formation.

The data of the alae formation and seam cell fusion were somewhat puzzling, considering that the double mutants appear to precociously express the *col-19::gfp* reporter. A potential explanation is that NHR-25 is also required for protein synthesis and/or secretion of COL-19 in addition to *col-19* expression. Therefore, we examined the protein level and distribution of the COL-19 proteins using an integrated array expressing COL-19::GFP translational fusion construct (Thein et al., 2003). Indeed, we observed no precocious the COL-19::GFP signals in *nhr-25(RNAi)* L4 animals (data not shown). This observation may imply the posttranscriptional regulation of COL-19 in the L/A transition.

lin-29 is required for *nhr-25* expression in the late L4 stage

During adult alae formation and seam cell fusion, the action of *nhr-25* resembles that of *lin-29,* which positively regulates the L/A transition (Rougvie, 2005; Rougvie and Ambros, 1995). We therefore examined whether there is a genetic interaction between *nhr-25* and *lin-29*. In experimental conditions using *nhr-25(RNAi)* bacteria diluted with an equal volume of mock RNAi to provide a lower intensity of RNAi, a small percentage of *nhr-25* or *lin-29* RNAi animals displayed retarded alae formation and

retarded seam cell fusion at the young adult stage (Fig. 6A, B). In contrast, we found a synergistic effect of *nhr-25* and *lin-29* on alae formation and seam cell fusion, as simultaneous RNAi against both *nhr-25* and *lin-29* with the same experimental condition gave rise to significant numbers of animals exhibiting retarded phenotypes in seam cells (Fig. 6A, B). This finding suggests that *nhr-25* and *lin-29* play overlapping roles in the same, or parallel, pathways to affect the L/A transition.

We also examined *nhr-25* expression levels using a transgenic line carrying a transcriptional *gfp* fusion with the *nhr-25* promoter (Silhankova et al., 2005). We found that *nhr-25::gfp* expression was moderately but significantly reduced in seam cells in *lin-29(RNAi)* animals in the late L4 stage but not earlier (Fig. 6C), suggesting that *lin-29* partially promotes *nhr-25* expression in seam cells.

nhr-25 and _hbl-1_ mutually inhibit each other in controlling seam cell proliferation

A more complicated genetic relationship between *nhr-25* and *hbl-1* was observed in developmental timing of seam cell division. As previously reported (Chen et al., 2004; Silhankova et al., 2005), *nhr-25(RNAi)* animals exhibited an increased number of seam cell nuclei as compared to wild-type animals at the adult stage (Figs. 7A, B and S9A, B). However, the number of seam cells was not increased in *nhr-25(RNAi)* animals in the early L4 stage in our conditions (Fig. S10A, B). These data suggest that NHR-25 normally represses seam cell proliferation in the L/A transition, and thus, the seam cell number defect of *nhr-25(RNAi)* young adults resembled the retarded *let-7* mutants (Reinhart et al., 2000). Similar to loss of *nhr-25* function worms, *hbl-1* mutations caused seam nuclei to divide inappropriately in the late L4, but not early L4 stages as also previously reported (Figs. 7C, S9C and S10C) (Abrahante et al., 2003; Lin et al., 2003). Unexpectedly, young adults with depletion of both *nhr-25* and *hbl-1* function

exhibited an almost normal seam cell number (Figs. 7D and S9D). This result implies that *nhr-25* and *hbl-1* mutually suppress each other to control seam cell proliferation in the L/A transition. Although the superficial phenotype of the loss of *nhr-25* or *hbl-1* function worm suggests at repressive role in seam cell division in the L/A transition, *nhr-25* and *hbl-1* are indeed positive regulators for seam cell division in the L/A transition.

Discussion

Identification of *nhr-25* as a heterochronic gene modulator

We show in this study that the evolutionarily conserved nuclear receptor NHR-25 (Asahina et al., 2000; Escriva et al., 1997; Gissendanner and Sluder, 2000) is required to regulate the L/A transition in *C. elegans*. Based upon the mixed phenotypes of loss of *nhr-25* function animals, which exhibit both retarded and precocious characteristics, we conclude that *nhr-25* is a novel heterochronic gene modulator essential for both promoting and preventing terminal differentiation of seam cells (Fig. 8). Our results imply that the L/A transition is not mediated by a simple cascade of heterochronic genes. Rather, each adult differentiation event is controlled by a cascade that has a genetic network distinct from other cascades, even though each cascade consists of similar sets of heterochronic genes. Thus, the molecular mechanism of developmental timing involves an unexpected complexity.

Previous studies have demonstrated that loss of *nhr-25* function causes various phenotypes throughout development, such as defects on embryogenesis, differentiation of larval epidermis, molting, distal tip cell/anchor cell formation, and cell-cell fusion in vulval morphogenesis (Asahina et al., 2000; Asahina et al., 2006; Chen et al., 2004;

Gissendanner and Sluder, 2000; Hajduskova et al., 2009; Silhankova et al., 2005). These observed phenotypes are the result of earlier or very severe knock-down and may have masked later or alternative functions. By contrast, our study clearly demonstrates genetic interactions between *nhr-25* and several heterochronic genes that have not previously been detected. It is possible that the penetrance and/or timing of the *nhr-25(RNAi)* in this work does not severely alter the vital development outcomes associated with earlier developmental defects that were previously reported (Asahina et al., 2000; Asahina et al., 2006; Chen et al., 2004; Gissendanner and Sluder, 2000; Hajduskova et al., 2009; Silhankova et al., 2005). We posit that the relatively weak RNAi conditions used in this study may have allowed us to uncover a novel developmental timing function of *nhr-25*. However, because this study involves hypomorphic alleles or RNAi of *hbl-1*, *lin-41*, *lin-42* and *nhr-25*, the interpretations of genetic relationships that we propose must be studied further.

Possible coupling of molecular mechanisms of developmental timing and molting

Molting is another major developmental timing landmark in the ecdysozoan life cycle. In addition to the essential role of *nhr-25* in the heterochronic gene network that we demonstrate in this study, *nhr-25* also has a function in controlling *C. elegans* molting (Asahina et al., 2000; Frand et al., 2005; Gissendanner and Sluder, 2000). We show that *apl-1*, known to be involved in *C. elegans* molting (Hornsten et al., 2007; Niwa et al., 2008), is necessary for the *nhr-25*-dependent regulation of molting. Our data suggest that the molting process at the L/A transition requires the *apl-1* expression in seam cells, in which a number of genes essential for molting are expressed (Frand et al., 2005). However, it has been shown that expression of *apl-1* in neuronal cells, rather than epidermal cells, is sufficient for the molting process at the L1/L2 transition (Hornsten et

al., 2007). Whereas the exact reason for the discrepancy is not yet understood, it is possible that the molting processes at different developmental stages need the proper expression of *apl-1* in different types of cells in *C. elegans*.

Known canonical heterochronic mutations affect neither larval growth nor progression through the molting cycle in *C. elegans*, even though individual hypodermal blast cells have the incorrect temporal identity (Ambros and Horvitz, 1984; Moss, 2007). Therefore, two independent timers have been proposed: one to control temporal boundaries of development such as molting and the other to regulate temporal identities such as stage-specific patterns of cell division and differentiation (Thummel, 2001). This view has been strengthened by recent reports that a combination of *let-7* family miRNA mutations exhibits only an extra molting phenotype but not an aberrant cell fate defect in the adult stage (Abbott et al., 2005; Hayes et al., 2006). Another piece of evidence supporting this view is that illegitimate activation of nicotinic receptor nAChRs during the second larval stage induces a lethal heterochronic phenotype by slowing developmental speed without affecting the molting timer (Ruaud and Bessereau, 2006). Controversially, our present study implies that one genetic component, *nhr-25*, participates in the regulation of both heterochronic temporal identity and molting in the L/A transition. More work is necessary to determine how the temporal identity conferred by the heterochronic genes is related to molting, for example through the identification of downstream target genes like *apl-1*.

NHR-25 can regulate the L/A transition in versatile ways

nhr-25 negatively regulates certain events in the L/A transition, such as expression of the adult-specific collagen gene *col-19* and seam cell division as well as larval molting programs in the adult stage (Hayes et al., 2006). In the same animals, *nhr-25* also

positively controls adult differentiation programs, including seam cell fusion and alae formation. Genetic relationships between *nhr-25* and other heterochronic genes are also diversified among adult differentiation programs, as summarized in Fig. 8.

How does NHR-25 accomplish these complicated interactions with other heterochronic genes (i.e., *hbl-1*, *lin-41*, and *lin-42*) to mediate the L/A transition within the same seam cells of the same animals? These interactions cannot be explained by simple temporal changes of transcriptional and/or translational regulation of identified heterochronic genes themselves, as *nhr-25* controls the developmental timing of *col-19* expression, seam cell division, seam cell fusion, and alae formation, all of which occur in the same seam cells. For example, although our data indicate that the *nhr-25* transcription level is influenced by the activity of *lin-29* (Fig. 6C), this effect can only account for the epistasis of *lin-29* to *nhr-25* for *apl-1* expression in the early L4 stage, alae formation, and seam cell fusion (Fig. 8C) and cannot explain the regulation of other adult differentiation programs (Fig. 8A1, A2, B). We also observed no obvious difference in GFP signals derived from *nhr-25::gfp::unc-54* transgenic lines between control and *hbl-1(RNAi)* animals (Fig. S11).

Alternatively, a possible explanation for the complexity of *nhr-25* genetic functions is that a specific protein could physically interact with NHR-25 to control each adult differentiation event. Like other NRs, the Ftz-F1/SF-1 family proteins physically interact with transcriptional coactivators and DNA-binding transcription factors (Pick et al., 2006). In *C. elegans*, direct physical and genetic interactions have been reported between NHR-25 and two Hox proteins, LIN-39 and NOB-1. Mutations in these genes display vulval phenotypes similar to *nhr-25* mutants (Chen et al., 2004). In the gonadal precursor cells, the physical interaction between NHR-25 and *C. elegans* β-catenins, WRM-1 and SYS-1, is important to modulate the Wnt/β-catenin signaling

(Asahina et al., 2006). Because the specific interaction between Ftz-F1/SF-1 and the binding partners may explain the spatial- and/or temporal-specific function of Ftz-F1/SF-1 (Pick et al., 2006), NHR-25 may have a certain binding partner that directs gene expression by itself and/or modulates the protein subcellular localizations for each adult differentiation program . Identifying potential binding partners for NHR-25 in seam cells would shed light on how NHR-25 has a variety of roles among different adult differentiation programs.

Insect Ftz-F1 and developmental timing

Interestingly, βFtz-F1, an insect ortholog of *nhr-25*, has been extensively studied in terms of how it regulates the timing of insect developmental processes such as molting and metamorphosis (Thummel, 2001). In insects, pulses of the steroid hormone ecdysone and its relative 20-hydroxyecdysone control transitions between life stages. These insects go through molting by activating stage-specific transcriptional cascades involving several NRs, including Ecdysone receptor and Ultraspiracle, which together form the receptor for 20-hydroxyecdysone, as well as DHR3, DHR38 and βFtz-F1 (Thummel, 2001). *nhr-23*, an ortholog of insect *DHR3*, and *nhr-25* have been directly linked with *C. elegans* molting (Asahina et al., 2000; Frand et al., 2005; Kostrouchova et al., 1998).

Drosophila ftz-f1 is also required for proper embryonic segmentation. Alternate segments are deleted in *ftz-f1* mutant embryos in a pair-rule fashion, as they are in mutants for the homeodomain protein Fushi tarazu (Ftz), with which Ftz-F1 interacts (Pick et al., 2006). Previous studies have indicated that spatial embryonic patterning in *Drosophila* segmentation and temporal patterning in seam cells of *C. elegans* share key conserved genes. For example, *C. elegans hbl-1* is an ortholog of *Drosophlila*

hunchback (*hb*). Interestingly, *hb* is best known for its essential role in spatial patterning in the *Drosophila* embryonic anterior and is also known to regulate temporal neuroblast identity (Isshiki et al., 2001; Rougvie, 2005). Furthermore, a recent study has shown that loss of *Drosophila squeeze*, a *Drosophila* homolog of the heterochronic gene *lin-29*, causes a temporal identity defect in *Drosophila* neuroblast (Tsuji et al., 2008). By analogy with these conserved heterochronic genes, it would be intriguing to investigate if *ftz-f1* is involved in the timing regulation of *Drosophila* neuroblast. It is noteworthy that *ftz* is expressed in a subset of *Drosophila* embryonic neurons (Doe, 1992).

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References

Abbott, A. L., Alvarez-Saavedra, E., Miska, E. A., Lau, N. C., Bartel, D. P., Horvitz, H.

R., Ambros, V., 2005. The *let-7* MicroRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. Dev. Cell. 9**,** 403-414.

Abrahante, J. E., Daul, A. L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A., Rougvie, A. E., 2003. The *Caenorhabditis elegans hunchback*-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. Dev. Cell. 4**,** 625-37.

Abrahante, J. E., Miller, E. A., Rougvie, A. E., 1998. Identification of heterochronic mutants in *Caenorhabditis elegans*. Temporal misexpression of a *collagen::green fluorescent protein* fusion gene. Genetics. 149**,** 1335-1351.

Ambros, V., 2000. Control of developmental timing in *Caenorhabditis elegans*. Curr. Opin. Genet. Dev. 10**,** 428-433.

Ambros, V., Horvitz, H. R., 1984. Heterochronic mutants of the nematode *Caenorhabditis elegans*. Science. 226**,** 409-16.

Asahina, M., Ishihara, T., Jindra, M., Kohara, Y., Katsura, I., Hirose, S., 2000. The conserved nuclear receptor Ftz-F1 is required for embryogenesis, moulting and reproduction in *Caenorhabditis elegans*. Genes Cells. 5**,** 711-723.

Asahina, M., Valenta, T., Silhankova, M., Korinek, V., Jindra, M., 2006. Crosstalk between a nuclear receptor and beta-catenin signaling decides cell fates in the *C.elegans* somatic gonad. Dev. Cell. 11**,** 203-211.

Banerjee, D., Kwok, A., Lin, S. Y., Slack, F. J., 2005. Developmental timing in *C. elegans* is regulated by *kin-20* and *tim-1*, homologs of core circadian clock genes. Dev. Cell. 8**,** 287-95.

Banerjee, D., Slack, F., 2002. Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. Bioessays.

24, 119-29.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D. J., Antebi, A., 2009. Nuclear hormone receptor regulation of microRNAs controls developmental progression. Science. 324, 95-98.

Bettinger, J. C., Lee, K., Rougvie, A. E., 1996. Stage-specific accumulation of the terminal differentiation factor LIN-29 during *Caenorhabditis elegans* development. Development. 122, 2517-2527.

Brooks, D. R., Appleford, P. J., Murray, L., Isaac, R. E., 2003. An essential role in molting and morphogenesis of *Caenorhabditis elegans* for ACN-1, a novel member of the angiotensin-converting enzyme family that lacks a metallopeptidase active site. J. Biol. Chem. 278, 52340-52346.

Chen, Z., Eastburn, D. J., Han, M., 2004. The *Caenorhabditis elegans* nuclear receptor gene *nhr-25* regulates epidermal cell development. Mol. Cell. Biol. 24, 7345-7358.

Doe, C. Q., 1992. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. Development. 116, 855-863.

Escriva, H., Safi, R., Hanni, C., Langlois, M. C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R., Laudet, V., 1997. Ligand binding was acquired during evolution of nuclear receptors. Proc Natl Acad Sci U S A. 94, 6803-8.

Esquela-Kerscher, A., Johnson, S. M., Bai, L., Saito, K., Partridge, J., Reinert, K. L., Slack, F. J., 2005. Post-embryonic expression of *C. elegans* microRNAs belonging to the *lin-4* and *let-7* families in the hypodermis and the reproductive system. Dev. Dyn. 234, 868-877.

Frand, A. R., Russel, S., Ruvkun, G., 2005. Functional genomic analysis of *C. elegans* molting. PLoS Biol. 3, e312.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., Ahringer, J., 2000. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature. 408**,** 325-30.

Gissendanner, C. R., Crossgrove, K., Kraus, K. A., Maina, C. V., Sluder, A. E., 2004. Expression and function of conserved nuclear receptor genes in *Caenorhabditis elegans*. Dev. Biol. 266**,** 399-416.

Gissendanner, C. R., Sluder, A. E., 2000. *nhr-25*, the Caenorhabditis elegans ortholog of *ftz-f1*, is required for epidermal and somatic gonad development. Dev. Biol. 221**,** 259-72.

Grosshans, H., Johnson, T., Reinert, K. L., Gerstein, M., Slack, F. J., 2005. The temporal patterning microRNA *let-7* regulates several transcription factors at the larval to adult transition in *C. elegans*. Dev. Cell. 8**,** 321-330.

Hajduskova, M., Jindra, M., Herman, M. A., Asahina, M., 2009. The nuclear receptor NHR-25 cooperates with the Wnt/beta-catenin asymmetry pathway to control differentiation of the T seam cell in *C. elegans*. J. Cell Sci. 122**,** 3051-3060.

Hammell, C. M., Karp, X., Ambros, V., 2009. A feedback circuit involving *let-7*-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA. 106**,** 18668-18673.

Hayes, G. D., Frand, A. R., Ruvkun, G., 2006. The *mir-84* and *let-7* paralogous microRNA genes of *Caenorhabditis elegans* direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25. Development. 133**,** 4631-41.

Hornsten, A., Lieberthal, J., Fadia, S., Malins, R., Ha, L., Xu, X., Daigle, I., Markowitz, M., O'Connor, G., Plasterk, R., Li, C., 2007. APL-1, a *Caenorhabditis elegans*

protein related to the human beta-amyloid precursor protein, is essential for viability. Proc. Natl. Acad. Sci. U.S.A. 104**,** 1971-6.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C. Q., 2001. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell. 106**,** 511-521.

Jeon, M., Gardner, H. F., Miller, E. A., Deshler, J., Rougvie, A. E., 1999. Similarity of the *C. elegans* developmental timing protein LIN-42 to circadian rhythm proteins. Science. 286**,** 1141-1146.

Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D., Slack, F. J., 2005. RAS is regulated by the let-7 microRNA family. Cell. 120**,** 635-47.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature. 421**,** 231-7.

Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J. E., 1998. CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. Development. 125**,** 1617-1626.

Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J. E., 2001. Nuclear hormone receptor CHR3 is a critical regulator of all four larval molts of the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA. 98**,** 7360-7365.

Lall, S., Grun, D., Krek, A., Chen, K., Wang, Y. L., Dewey, C. N., Sood, P., Colombo, T., Bray, N., Macmenamin, P., Kao, H. L., Gunsalus, K. C., Pachter, L., Piano, F., Rajewsky, N., 2006. A genome-wide map of conserved microRNA targets in *C. elegans*. Curr. Biol. 16**,** 460-471.

Li, M., Jones-Rhoades, M. W., Lau, N. C., Bartel, D. P., Rougvie, A. E., 2005. Regulatory mutations of *mir-48*, a *C. elegans let-7* family MicroRNA, cause developmental timing defects. Dev Cell. 9**,** 415-422.

Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., Gamberi, C., Gottlieb, E., Slack, F. J., 2003. The *C. elegans hunchback* homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. Dev. Cell. 4**,** 639-50.

Magner, D. B., Antebi, A., 2008. *Caenorhabditis elegans* nuclear receptors: insights into life traits. Trends Endocrinol. Metab. 19**,** 153-160.

Moss, E. G., 2007. Heterochronic genes and the nature of developmental time. Curr. Biol. 17**,** R425-R434.

Niwa, R., Zhou, F., Li, C., Slack, F. J., 2008. The expression of the Alzheimer's *amyloid precursor protein-like* gene is regulated by developmental timing microRNAs and their targets in *Caenorhabditis elegans*. Dev. Biol. 315**,** 418-425.

Ong, K. K., Elks, C. E., Li, S., Zhao, J. H., Luan, J., Andersen, L. B., Bingham, S. A., Brage, S., Smith, G. D., Ekelund, U., Gillson, C. J., Glaser, B., Golding, J., Hardy, R., Khaw, K. T., Kuh, D., Luben, R., Marcus, M., McGeehin, M. A., Ness, A. R., Northstone, K., Ring, S. M., Rubin, C., Sims, M. A., Song, K., Strachan, D. P., Vollenweider, P., Waeber, G., Waterworth, D. M., Wong, A., Deloukas, P., Barroso, I., Mooser, V., Loos, R. J., Wareham, N. J., 2009. Genetic variation in *LIN28B* is associated with the timing of puberty. Nat. Genet. 41**,** 843-848.

Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C., Ball, E. E., Degnan, B., Muller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E.,

Ruvkun, G., 2000. Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. Nature. 408**,** 86-89.

Pick, L., Anderson, W. R., Shultz, J., Woodard, C. T., 2006. The Ftz-F1 family: Orphan nuclear receptors regulated by novel protein-protein interactions. Adv. Dev. Biol. 16**,** 255-296.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R., Ruvkun, G., 2000. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. Nature. 403**,** 901-906.

Rougvie, A. E., 2005. Intrinsic and extrinsic regulators of developmental timing: from miRNAs to nutritional cues. Development. 132**,** 3787-3798.

Rougvie, a. E., Ambros, V., 1995. The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. Development. 121**,** 2491-2500.

Roush, S. F., Slack, F. J., 2009. Transcription of the *C. elegans let-7* microRNA is temporally regulated by one of its targets, *hbl-1*. Dev. Biol.

Ruaud, A. F., Bessereau, J. L., 2006. Activation of nicotinic receptors uncouples a developmental timer from the molting timer in *C. elegans*. Development. 133**,** 2211-2222.

Silhankova, M., Jindra, M., Asahina, M., 2005. Nuclear receptor NHR-25 is required for cell-shape dynamics during epidermal differentiation in *Caenorhabditis elegans*. J. Cell Sci. 118**,** 223-232.

Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R., Ruvkun, G., 2000. The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. Mol. Cell. 5**,** 659-69.

Sulston, J. E., Horvitz, H. R., 1977. Post-embryonic cell lineages of the nematode

Caenorhabditis elegans. Dev. Biol. 56**,** 110-156.

Tennessen, J. M., Gardner, H. F., Volk, M. L., Rougvie, A. E., 2006. Novel heterochronic functions of the *Caenorhabditis elegans* period-related protein LIN-42. Dev. Biol. 289**,** 30-43.

Thein, M. C., McCormack, G., Winter, A. D., Johnstone, I. L., Shoemaker, C. B., Page, A. P., 2003. *Caenorhabditis elegans* exoskeleton collagen COL-19: an adult-specific marker for collagen modification and assembly, and the analysis of organismal morphology. Dev. Dyn. 226**,** 523-539.

Thummel, C. S., 2001. Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Dev. Cell. 1**,** 453-465.

Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature. 395**,** 854.

Tsuji, T., Hasegawa, E., Isshiki, T., 2008. Neuroblast entry into quiescence is regulated intrinsically by the combined action of spatial Hox proteins and temporal identity factors. Development. 135**,** 3859-3869.

Figure Legends

Fig. 1. *nhr-25* is required for *apl-1* expression in seam cells.

All worms shown in this figure were carrying an integrated array of *apl-1::gfp::unc-54* constructs fused with the full 7.0 kb *apl-1* promoter sequence. The GFP signals in seam cells are marked by arrowheads in A, C, E, and G. In DIC images, arrows and arrowheads point to gonadal distal tip cells and vulva, respectively. (A-C) Late L4 larvae carrying integrated *apl-1::gfp::unc-54* constructs on RNAi plates of control (mock, A), *nhr-25* (B) and *nhr-23* (C). Images A-C were taken for the same exposure time and processed identically. (D) Temporal expression profiles of the *apl-1::gfp::unc-54* strains with control (mock) RNAi, *nhr-25* RNAi, *nhr-23* RNAi, *daf-12* RNAi, and *nhr-25(ku217)* mutation. (E-G) Precocious (early L4) *apl-1::gfp* expression in seam cells in *hbl-1(ve18)* mutant with RNAi for control (mock, E), *nhr-25* (F), and *nhr-23* (G). Images E-G were taken for the same exposure time and processed identically. (H) Percentage of animals showing *apl-1::gfp::unc-54* expression in the wild type, *hbl-1* and *lin-42* backgrounds with mock RNAi , *nhr-25* RNAi and *nhr-23* RNAi in the early L4 stage. Open bars indicate that no GFP signal in seam cells was observed, shaded bars indicate that GFP signals were observed in some seam cells, and dark bars indicate that GFP signals were observed in all seam cells. Each number in parentheses presents the number (n) of observed animals in each experiment. Scale bar: 100 μm.

Fig. 2. *nhr-25* genetically interacts with *apl-1* to regulate molting.

(A) *nhr-25(ku217)* animal was fed *apl-1* RNAi bacteria from L1 stage. Unshed cuticle was observed at the L4 molt (arrows). Scale bar: 200 μm. (B) Percentage of animals

showing the double cuticle phenotype at the L4 molt in *nhr-25* mutant with mock RNAi or *nhr-25(RNAi)*. Each number in parentheses indicates the total number of observed animals in each experiment. ***p<0.001 chi-square test.

Fig. 3. *nhr-25* acts synergistically with *hbl-1, lin-41, and lin-42* to cause precocious *col-19* expression.

(A-E) Animals showing adult specific collagen *col-19::gfp* expression in the L4 stage of wild type (A-C) and *hbl-1(mg285)* mutants (D, E) with control (mock) RNAi (A, C) and *nhr-25* RNAi (B, C, E). Mid L4 animals are shown in A and B, and early L4 animals are shown in C-E. Each box in the DIC image indicates the head region where the *col-19::gfp* signal is represented. In DIC images, arrows and arrowheads point to gonadal distal tip cells and vulva, respectively. Scale bar: 30 μm for GFP images and 100 μm for DIC images. (F) Percentage of animals showing precocious *col-19::gfp* expression in mid L4 stage in the heterochronic mutants and *nhr-25 (ku217)* mutant. (G) Percentage of animals showing *col-19::gfp* expression in the early L4 stage in heterochronic mutants with mock RNAi or *nhr-25* RNAi. Each number in parentheses indicates the total number of observed animals in each experiment. ***p<0.001 chi-square test.

Fig. 4. Precocious alae formation is suppressed by *nhr-25* RNAi in the heterochronic mutants.

(A-C) Scanning electron micrographs of precocious alae in early L4 animals of wild type N2 with control (mock) RNAi (A), *hbl-1(ve18)* mutant with mock RNAi (B) and *hbl-1(ve18)* mutant with *nhr-25* RNAi (C) in the early L4 stage. In wild type, adult alae were not observed in L4 animals (A). The precocious alae in *hbl-1* mutant (B) were

suppressed by *nhr-25* RNAi (C). Arrowheads indicate alae. Scale bar: 5 μm. (D) Percentage of animals showing precocious alae formation in the L4 stage. Each number in parentheses indicates the number (n) of observed animals in each sample. ***$p<0.001$ chi-square test.

Fig. 5. Precocious seam cell fusion is suppressed by *nhr-25* RNAi in the heterochronic mutants.

Seam cell junctions were visualized in the integrated GFP strain *wIs79* containing both *ajm-1::gfp* and *scm::gfp*. (A) *hbl-1(mg285); wIs79* animals had precocious fused seam cells in the early L4 stage as indicated by lack of AJM-1::GFP signal at cell junctions. (B) *hbl-1 (mg285); nhr-25 RNAi* animals had unfused seam cells in the early L4 stage as indicated by GFP signal at the cell junctions (arrows). Inset is a higher magnification picture corresponding to the region marked by dashed box. Scale bar: 100 μm. (C) Temporal profiles of seam cell fusion in the L4 stage in heterochronic mutants with or without *nhr-25* RNAi. Open bars indicate that any fusion in seam cells was not observed, shaded bars indicate that some seam cells fused, and dark bars indicate that all seam cells fused. Each number in parentheses presents the number (n) of each sample.

Fig. 6. *nhr-25* interacted with and is in part transcriptionally regulated by *lin-29*.

(A, B) Synergistic genetic interactions between *nhr-25* and *lin-29* for controlling adult alae formation and seam cell fusion. Percentages of animals without complete, normal alae structure (A) and of animals not showing normal seam cell fusion (B) in *nhr-25* RNAi, *lin-29* RNAi and *lin-29;nhr-25* double RNAi animals at young adult stages. For B, cell junctions were visualized using the *wIs79* strain. Each number in parentheses presents the number of each sample. *nhr-25* and *lin-29* RNAi bacteria were doubly

diluted with an equal volume of mock RNAi to provide a lower intensity of RNAi. To statistically examine a synergistic interaction between *nhr-25* and *lin-29*, chi-square test was performed comparing between data of *lin-29;nhr-25* double RNAi and a summation of data of *nhr-25* RNAi alone and *lin-29* RNAi alone. (C) Temporal expression profiles of the extrachromosomal *nhr-25::gfp::unc-54* lines with control (mock) and *lin-29* RNAi in early, mid, and late L4 stages. *nhr-25* expression in seam cells was significantly reduced in *lin-29* RNAi animals in the late L4 stage, but not earlier. ***$p<0.001$ chi-square test.

Fig. 7. *nhr-25* acts with *hbl-1* to control seam cell division.

Seam cell nuclei were visualized and scored by an integrated GFP strain *wIs51[scm::gfp]*. Seam cell nuclei were counted on one side of animals at the young adult stage (48 hours after hatching). Average numbers of seam cells (± S.D.) are also shown. X-axis is the number of seam cell nuclei, and y-axis is the number of animals. Each genotype is indicated in its panel.

Fig. 8. *nhr-25* has a dual role in regulating the L/A transition.

nhr-25 negatively regulates *apl-1* expression at the L/A transition (A-1, A-2). Our data suggest that *nhr-25* regulates *apl-1* downstream of *hbl-1*, *lin-41* and *lin-42* in the early L4 stage (A-1), while *nhr-25* regulates *apl-1* in parallel to these heterochronic genes in the late L4 stage (A-2). The *nhr-25*-mediated *apl-1* expression in the late L4 stage is required for molting at the L/A transition. *nhr-25* also negatively regulates *col-19* expression in the L4 stage, while *nhr-25* seems to positively regulate *col-19* expression in the adult stage (B). At the same time, *nhr-25* also positively regulates alae formation and seam cell fusion at the L/A transition. (C). In this case, *nhr-25* is activated by *lin-29*

in part at the transcription level. However, in the case of D, *nhr-25* and *hbl-1* mutually suppress each other to control seam cell proliferation during the L/A transition, whereas *nhr-25* and *hbl-1* themselves indeed are positive regulators of seam cell division at the L/A transition (D). It is noteworthy that the epistatic relationships between *nhr-25* and other heterochronic genes are diverse among adult differentiation programs.

Figure 1
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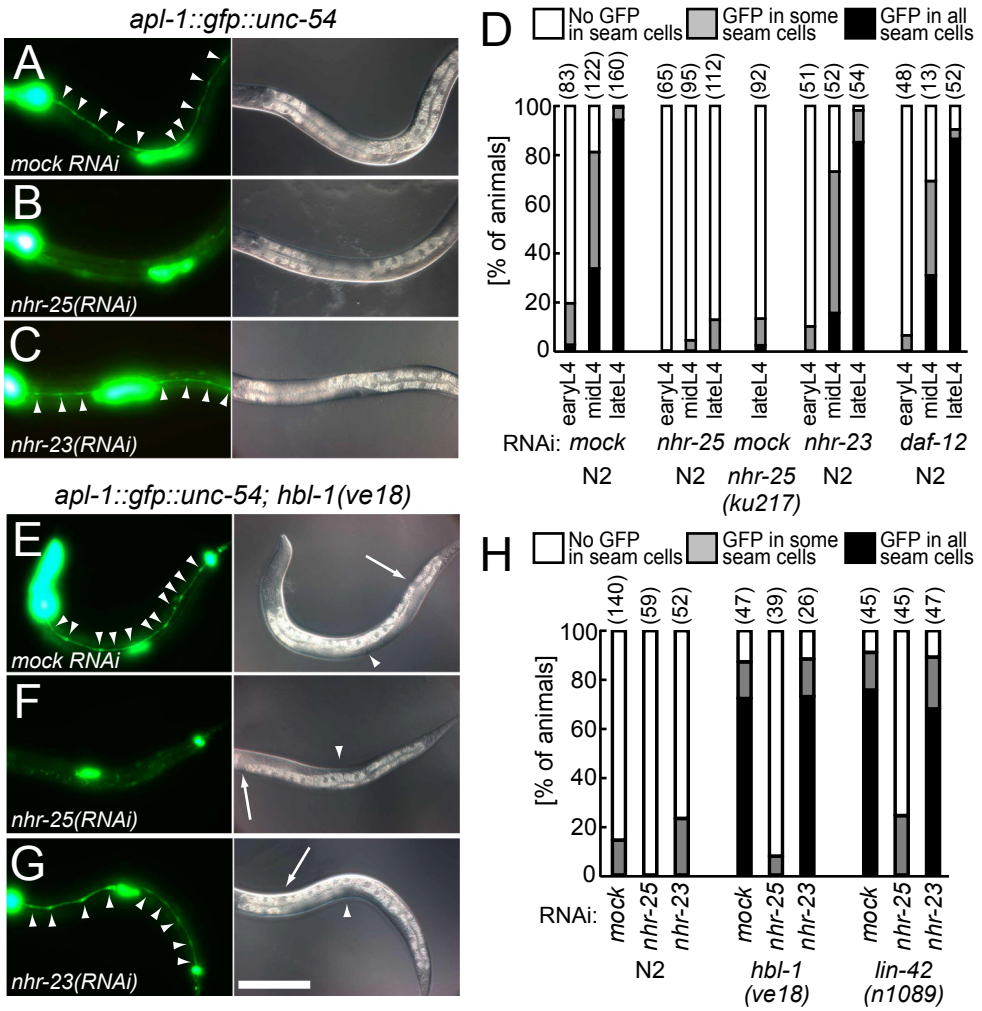


Figure 2
Hada et al.

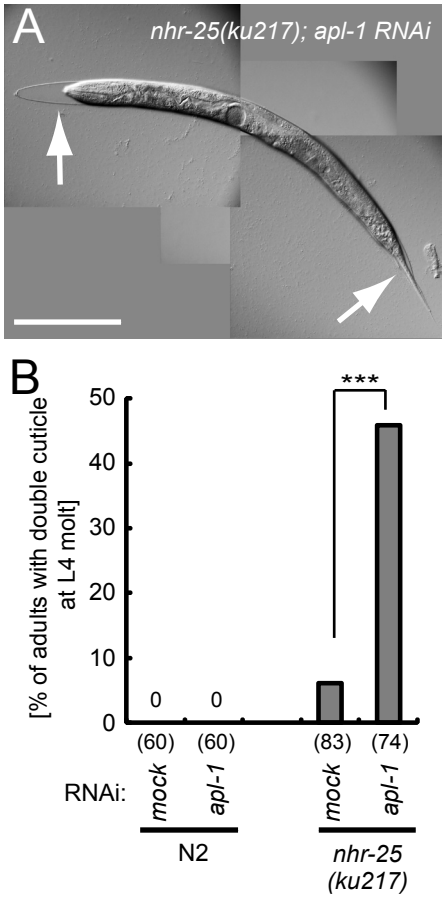


Figure 3
Hada et al.

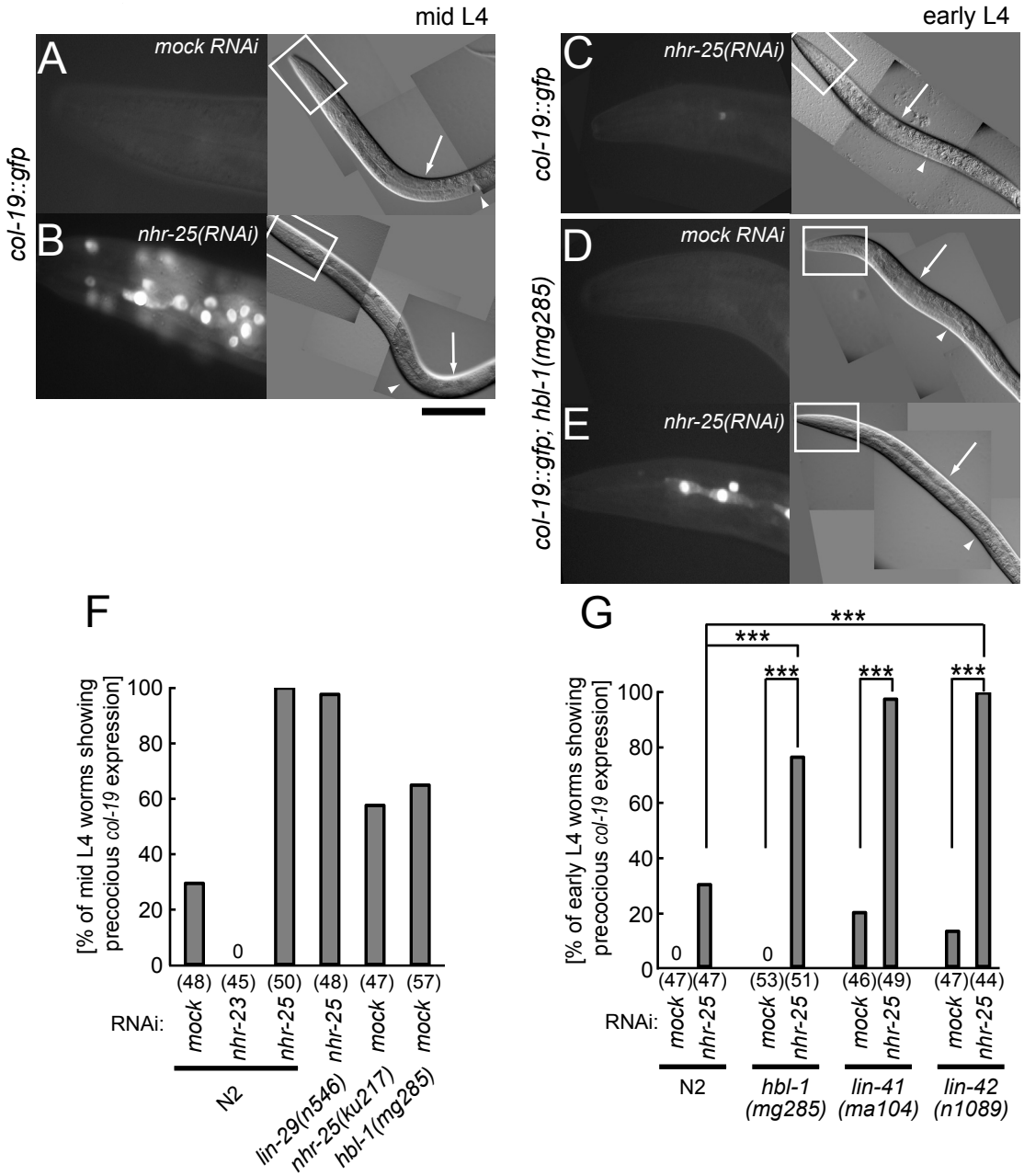


Figure 4
Hada et al.

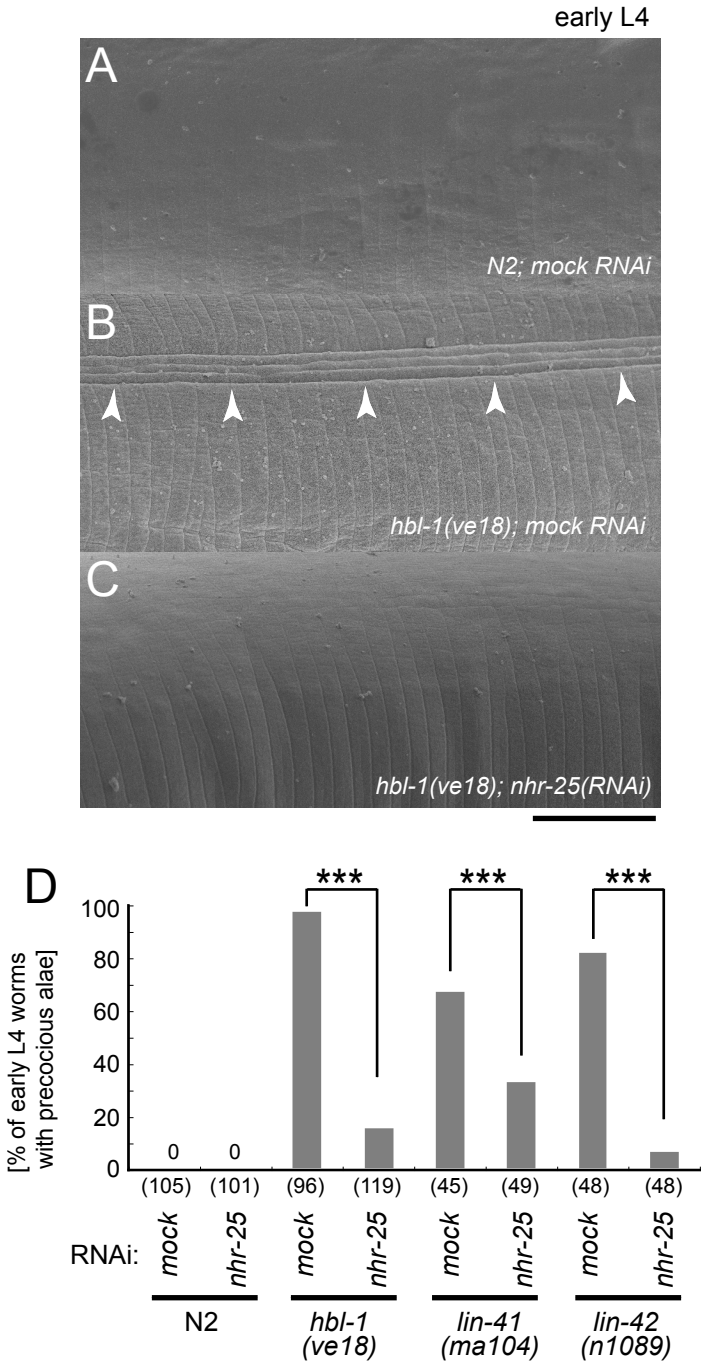


Figure 5
Hada et al.

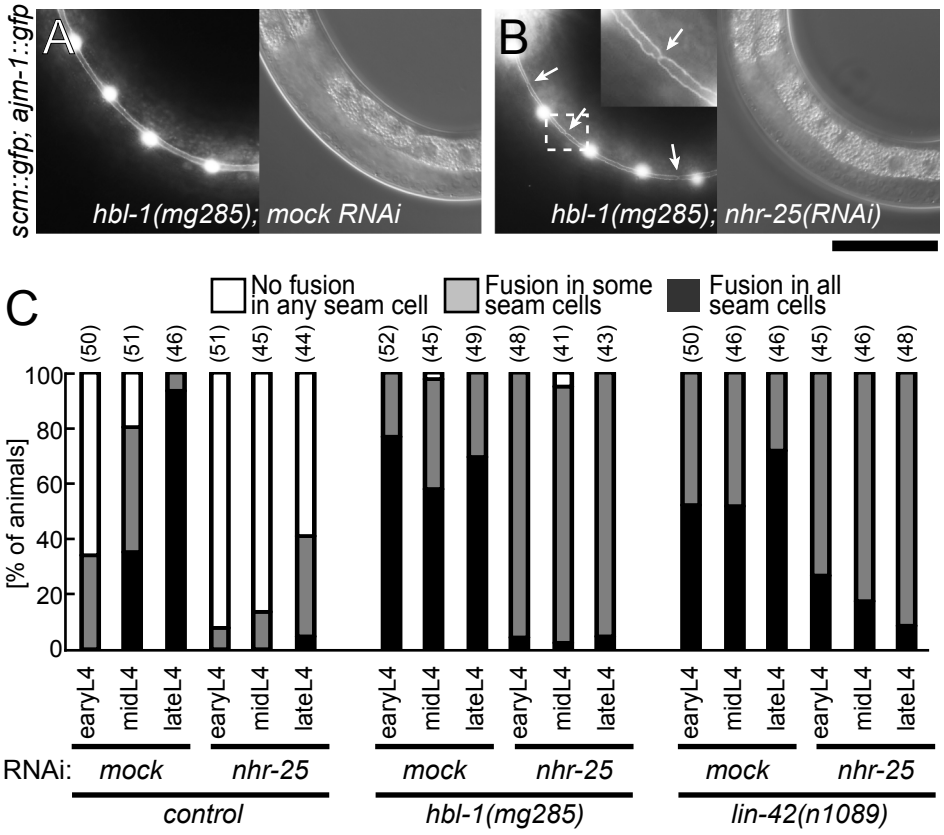


Figure 6
Hada et al.

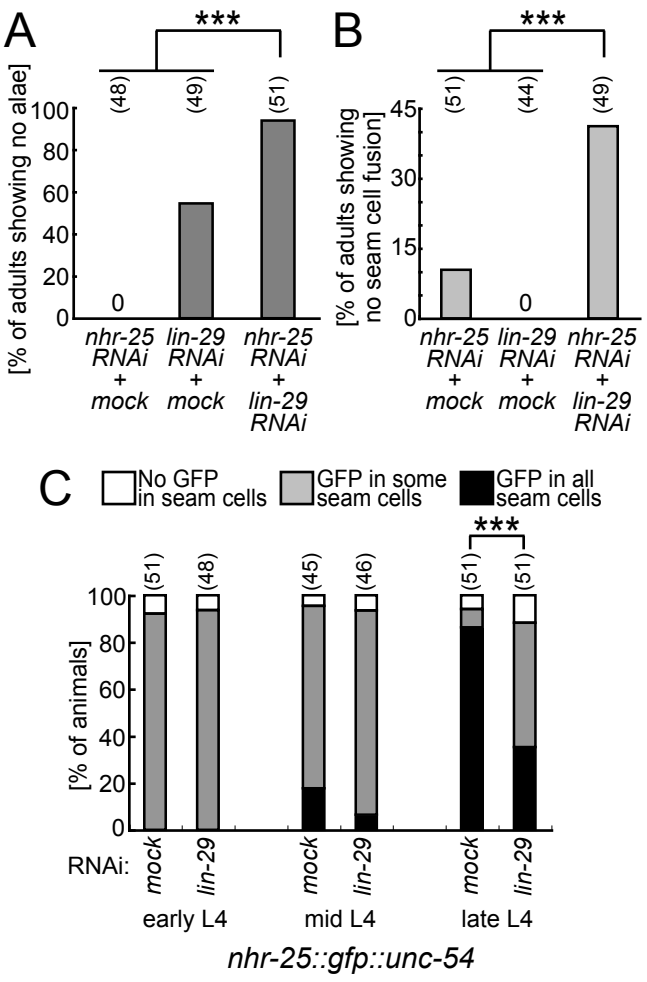


Figure 7
Hada et al.

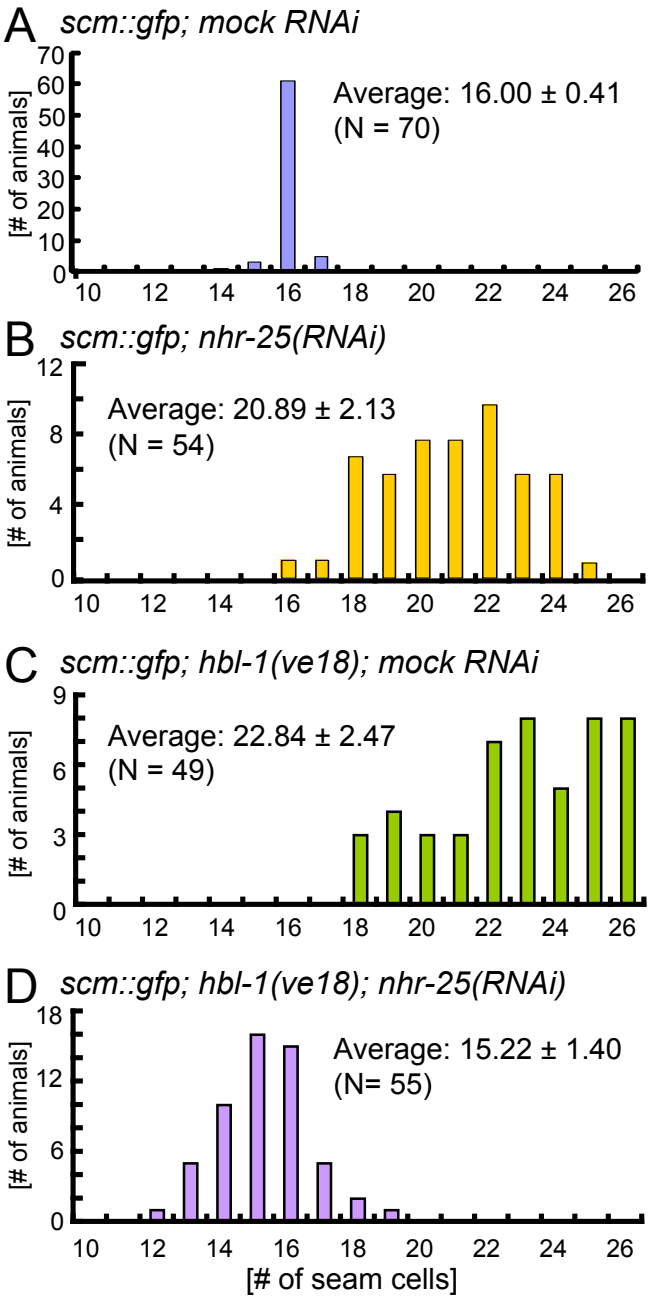
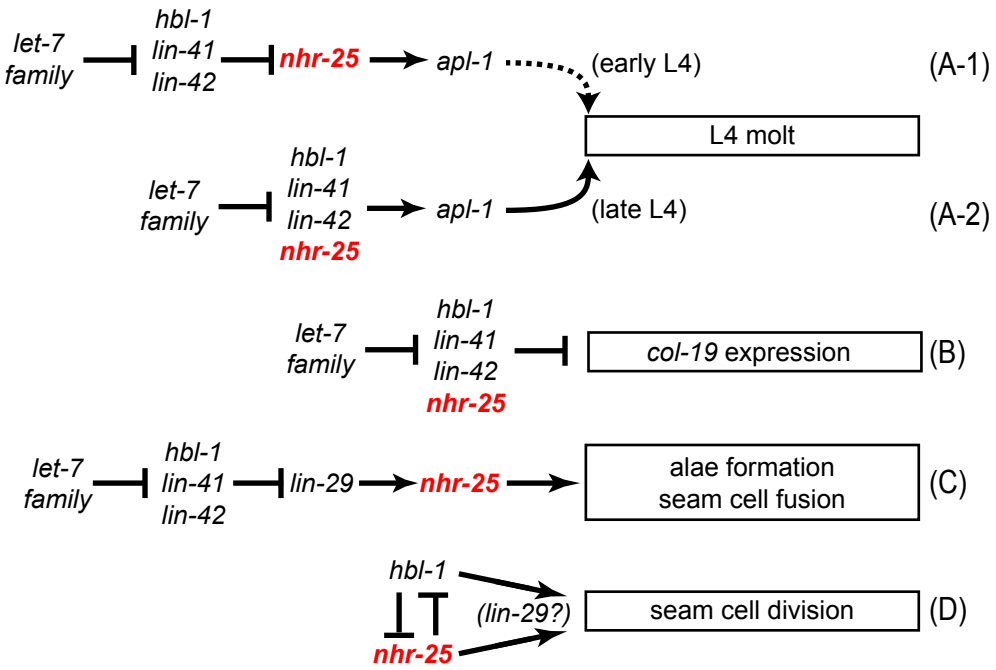


Figure 8
Hada et al.



Supplementary Figure Legends

Fig. S1. Developmental profile of *apl-1* expression in seam cells.

All worms shown in this figure were carrying an integrated array of *apl-1::gfp::unc-54* constructs fused with the full 7.0 kb *apl-1* promoter sequence. In each panel, GFP signal (left) and DIC image (right) were shown. Each white box in DIC image indicates the region where GFP image was taken. Stages are late L1 (A), early L2 (B), late L2 (C), early L3 (D), late L3 (E), early L4 (F) and late L4 (G). *apl-1* expression in seam cells are marked by arrowheads. The strong expression was observed only in the late L4 stage (G). While faint expressions were frequently observed in the L2 and L3 stages (C, E), the penetrance was low (<10%). Scale bars: 50 μm.

Fig. S2. High magnification of seam cells in the *apl-1::gfp::unc-54* strain

Also see Fig. 1. Each white box in DIC image indicates the region where GFP image was taken. Arrowheads indicate *apl-1* expression in seam cells. (A, B) Late L4 larvae carrying integrated *apl-1::gfp::unc-54* constructs on RNAi plates of control (mock, A) and *nhr-25* (B). Images A and B were taken for the same exposure time and processed identically. (C, D) Precocious (early L4) *apl-1::gfp* expression in seam cells in *hbl-1(ve18)* mutant with RNAi for control (mock, C) and *nhr-25* (F). Images C and D were taken for the same exposure time and processed identically. Scale bars: 50 μm.

Fig. S3. *apl-1* expression in pharyngeal cells and uterine cells.

(A, B) Late L4 larvae carrying integrated *apl-1::gfp::unc-54* constructs on RNAi plates of control (mock, A) and *nhr-25* (B). GFP images were taken in shorter exposure time as compared to Fig. 1A-C. The GFP signals in pharyngeal cells and uterine cells are

marked by arrowheads and arrows, respectively. Scale bar: 100 μm. (C) Quantification of GFP levels (mean±SEM) in pharyngeal muscles and uterine pi cells of *apl-1::gfp::unc-54* in seam cells in control (mock) and *nhr-25(RNAi)* late L4 worms. $^{**}p<0.01$ Student's *t*-test. Each number in parentheses indicates n of observed seam cells of each sample.

Fig. S4. *daf-12* is not involved in regulating *apl-1* expression.

apl-1 expression in seam cells in control (mock) (A) and *daf-12* RNAi worms (B) in the late L4 stage as judged from the shape of gonad and Christmas tree-like vulvas. Left: GFP signals, right: DIC images. The integrated *apl-1::gfp::unc-54* line was used. Seam cells are marked by arrowheads. Scale bar: 100 μm.

Fig. S5. Genetic interactions of *lin-29* with *apl-1* and *nhr-25* on the regulation of molting at the L4 molt.

Percentage of animals showing the double cuticle phenotype at the L4 molt in N2 wild type and *lin-29* mutant animals with mock RNAi, *apl-1(RNAi)* and *nhr-25(RNAi)*. Each number in parentheses indicates the total number of observed animals in each experiment.

Fig. S6. The weak expression of *col-19::gfp* in the late L4 stage in wild type background.

Animals showing adult specific collagen *col-19::gfp* expression in the late L4 stage of wild type with mock RNAi. A box in the DIC image indicates the head region where the *col-19::gfp* signal is represented. GFP images with the short (left) and long (middle) exposures are shown. The exposure time of the shorter photograph was identical to that

of photographs of Fig. 3A-E. The photograph with longer exposure shows the weak *col-19::gfp* expression. In DIC images, arrows and arrowheads point to gonadal distal tip cells and vulva, respectively. Scale bar: 30 μm for GFP images and 100 μm for the DIC image.

Fig. S7. *nhr-25*, but not the *nhr-23*, is involved in regulating *col-19* expression.
(A) The *col-19::gfp* expression in *nhr-25(ku217)* mutant precociously expressed in seam cells. This is consistent with the results of *nhr-25* RNAi animals shown in Fig. 3. (B) The *col-19::gfp* expression in *nhr-23* RNAi mutants was not observed in mid-late L4 stage. Scale bar: 100 μm.

Fig. S8. The shape of seam cells in the *nhr-25* RNAi mutants is abnormal.
Seam cell junctions and nuclei were visualized by an integrated GFP strain *wIs79* containing *ajm-1::gfp* and *scm::gfp*, respectively. Arrowheads in GFP panels indicate cell junctions between seam cells. Arrows and arrowheads in DIC panels point to distal gonad tips and vulvae, respectively. (A) *wIs79* animal with mock RNAi had unfused seam cells in early L4 stage. (B) *wIs79; nhr-25(RNAi)* animals still kept unfused seam cells even in the late L4 stages. Arrows in GFP panel indicate gaps between seam cells. Scale bar: 50 μm.

Fig. S9. Seam cell numbers in wild type and heterochronic mutant animals in early L4 stage.
Quantification of seam cell numbers were performed visualizing seam nuclei by an integrated GFP strain *wIs51* containing *scm::gfp*. Seam nuclei were counted on one side of animals in early L4 stage. An x-axis represents number of seam cells, and a y-axis

represents number of animals. Average numbers of seam cells (± S.D.) are also shown. (A) Control animal, (B) *nhr-25(RNAi)*, (C) *hbl-1(ve18)*, and (D) *hbl-1(ve18);nhr-25(RNAi)* animals.

Fig. S10. Seam cells in wild type and heterochronic mutant animals in late L4 stage.

Seam cell nuclei were visualized and scored by an integrated GFP strain *wIs51[scm::gfp]*. Fluorescence images of SCM::GFP signals in control RNAi (A), *nhr-25(RNAi)* (B), *hbl-1(ve18)* (C), and *hbl-1(ve18); nhr-25(RNAi)* (D) animals. Scale bar: 100 μm.

Fig. S11. *nhr-25* expression in the L4 stage

GFP signals from an extrachromosomal array of *nhr-25::gfp::unc-54* in the mock *RNAi* and *hbl-1* RNAi late L4 larvae. GFP images were taken for the same exposure time and processed identically.

Figure S1
Hada et al.

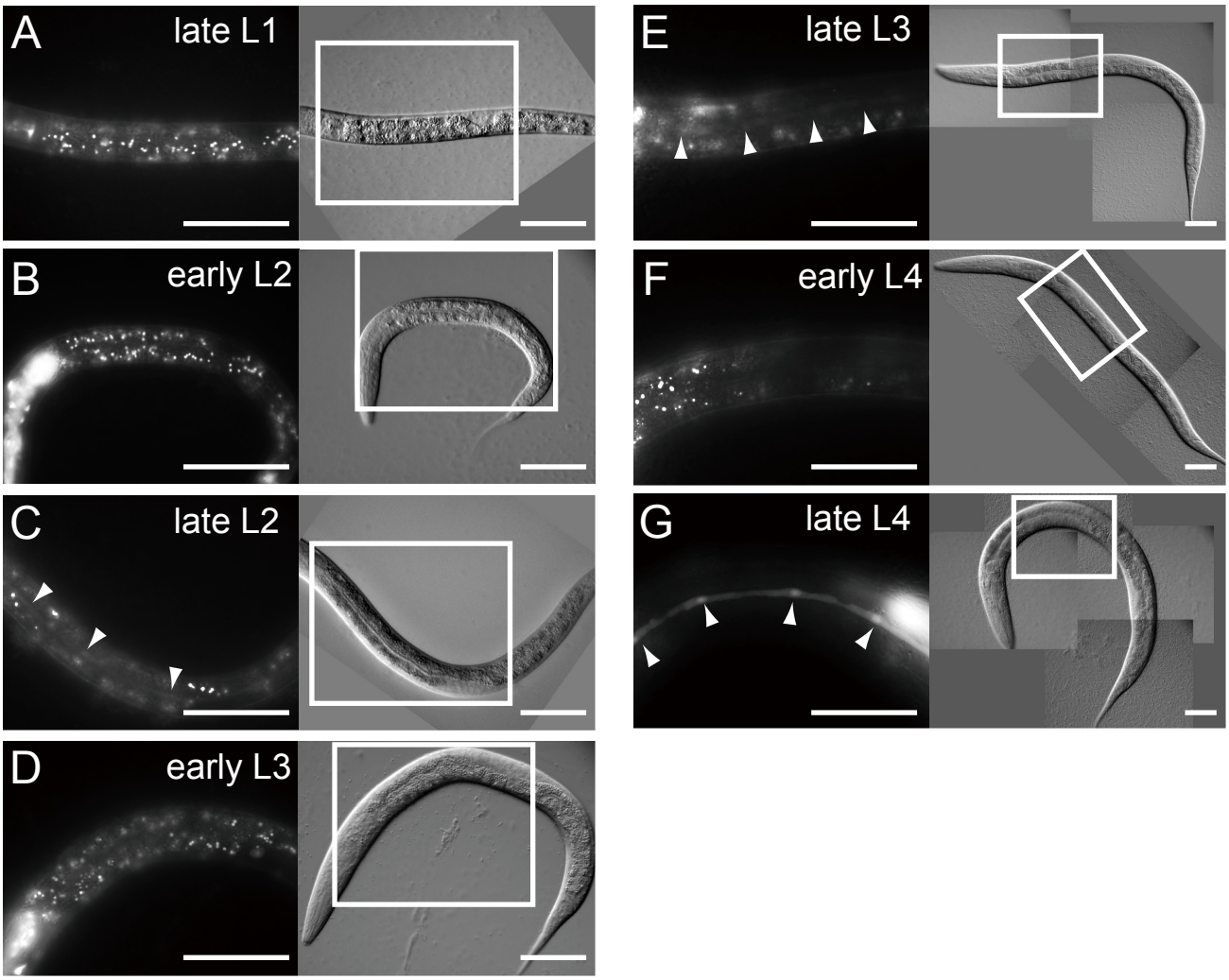


Figure S2
Hada et al.

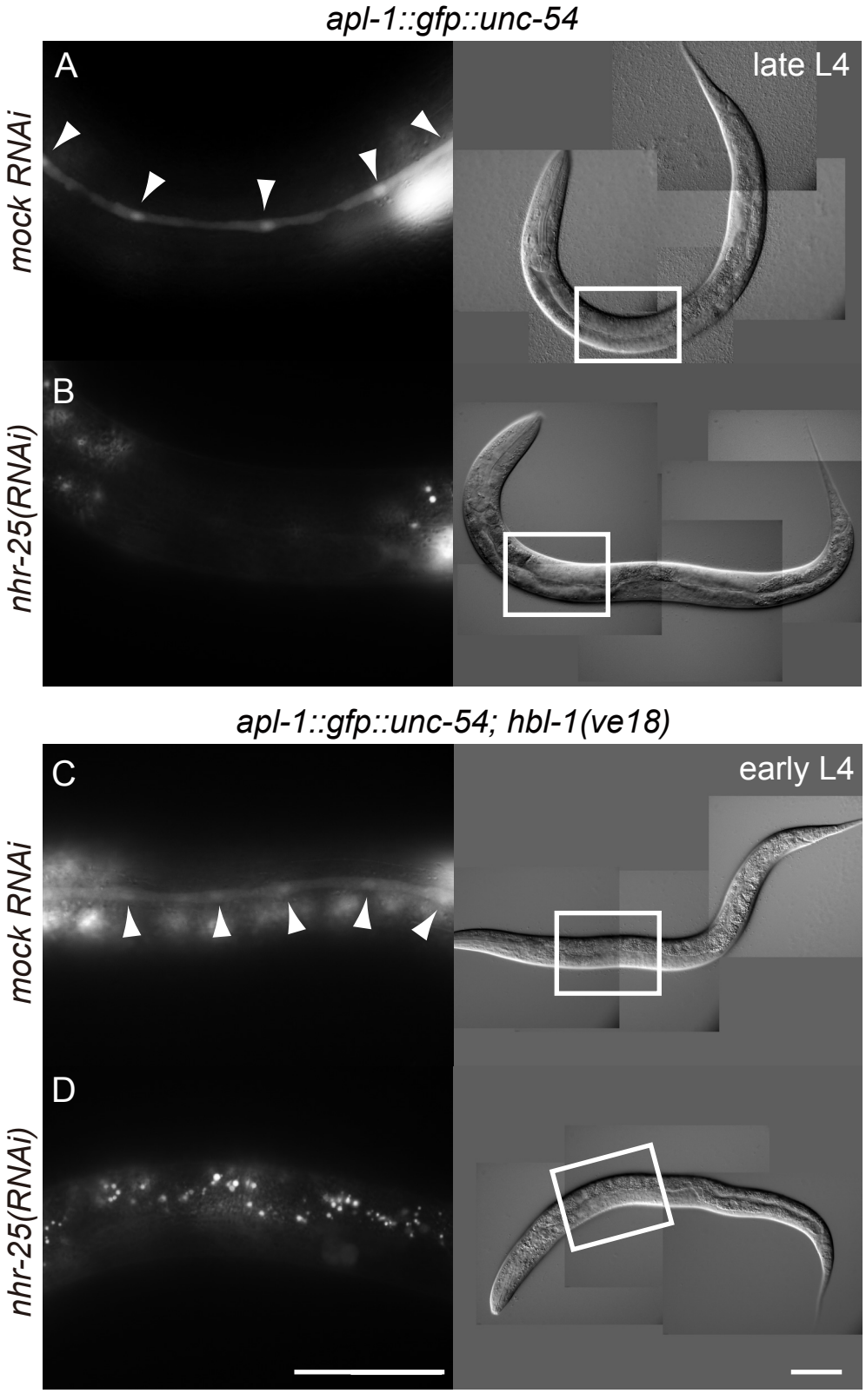
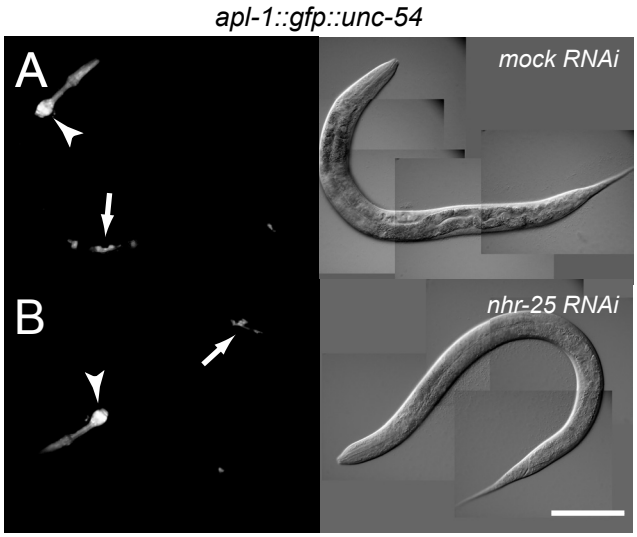


Figure S3
Hada et al.



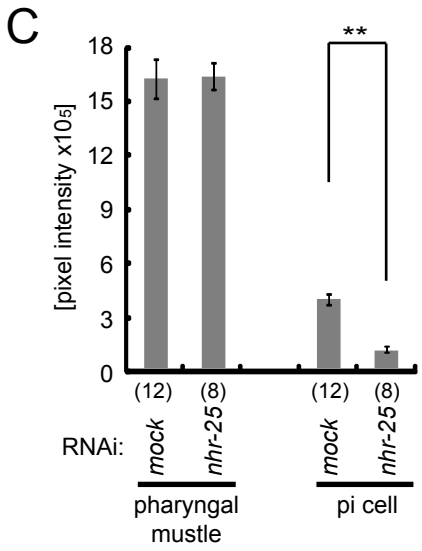


Figure S4
Hada et al.

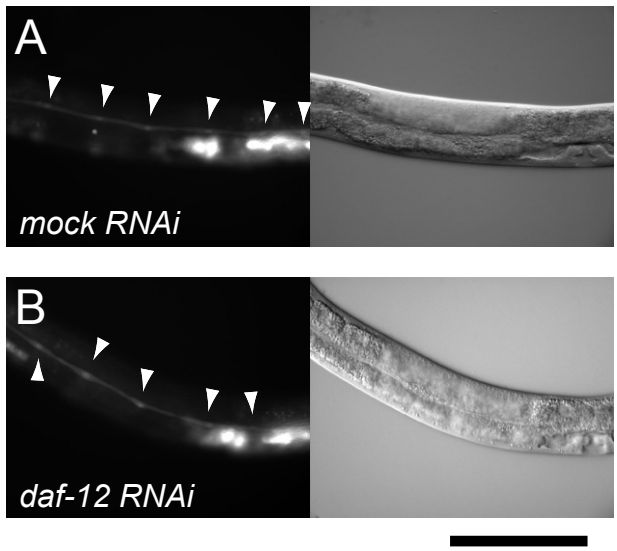
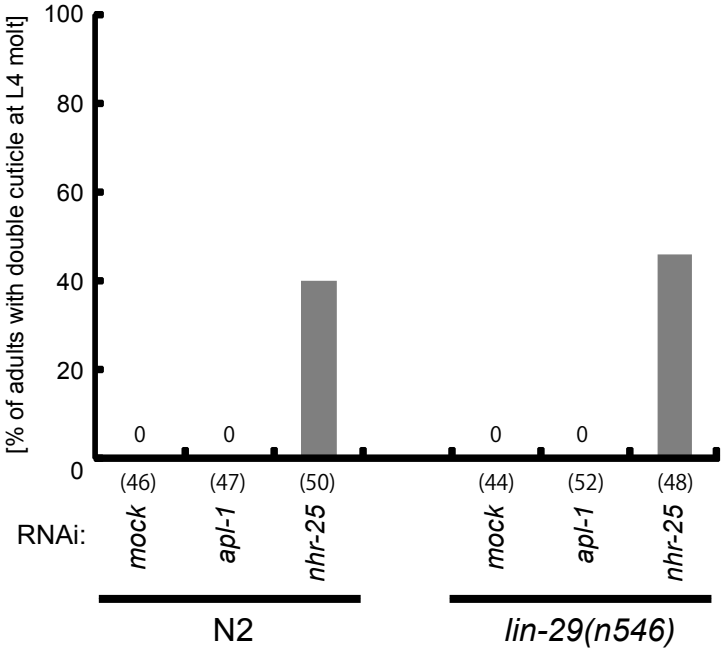


Figure S5
Hada et al.



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Hada et al.

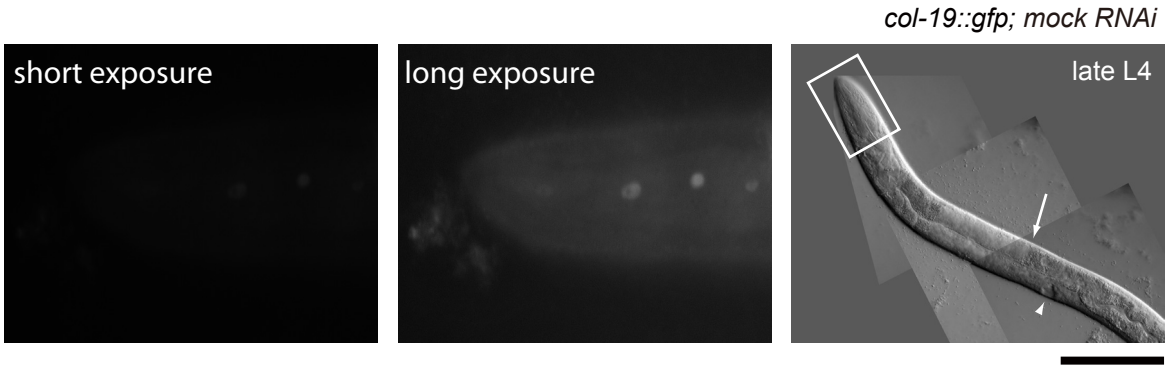


Figure S7
Hada et al.

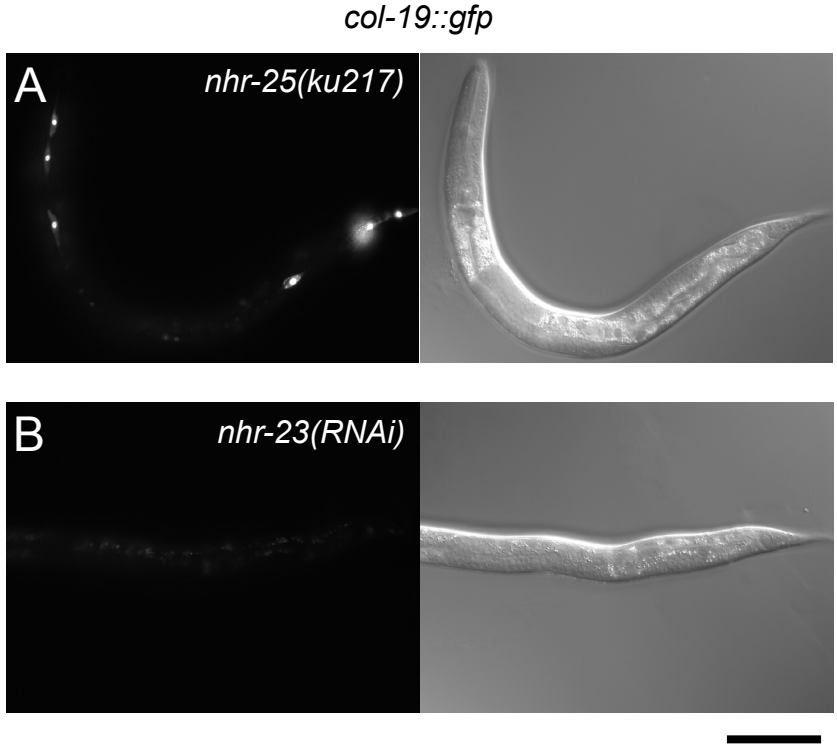


Figure S8
Hada et al.

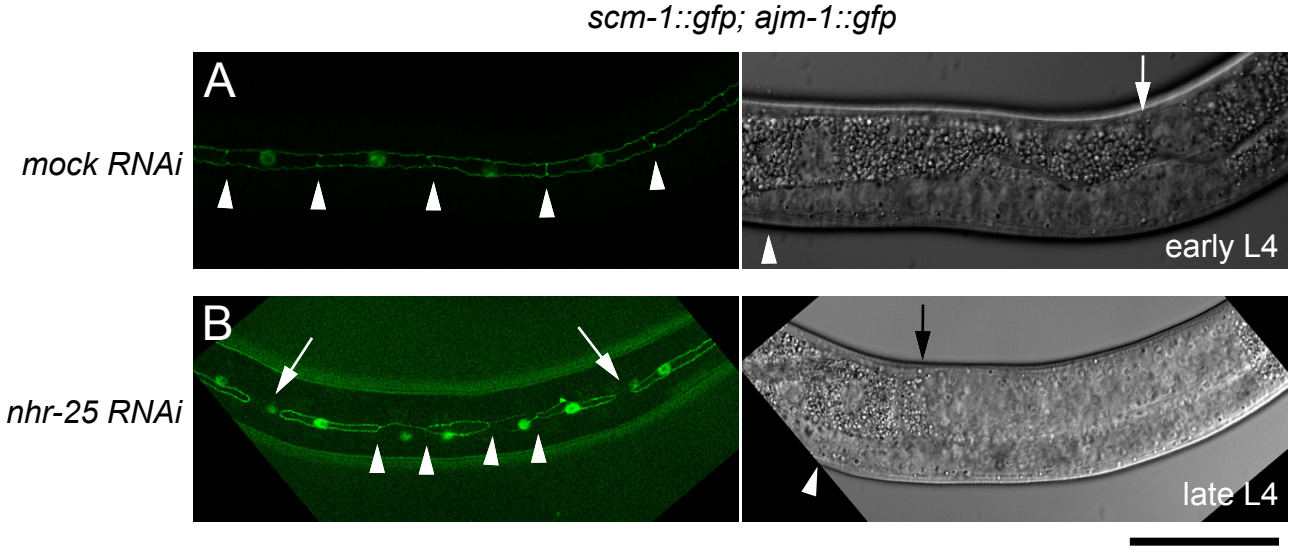
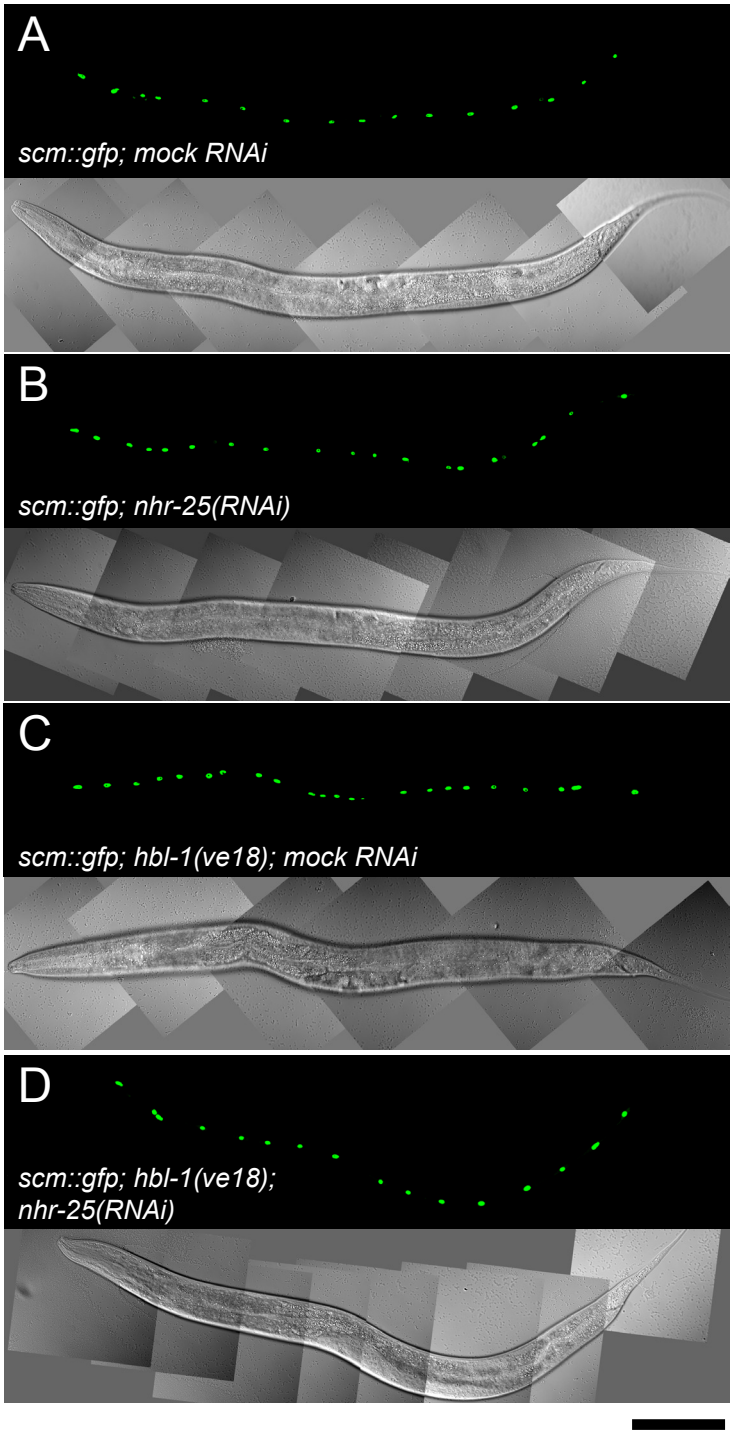


Figure S9
Hada et al.



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Hada et al.

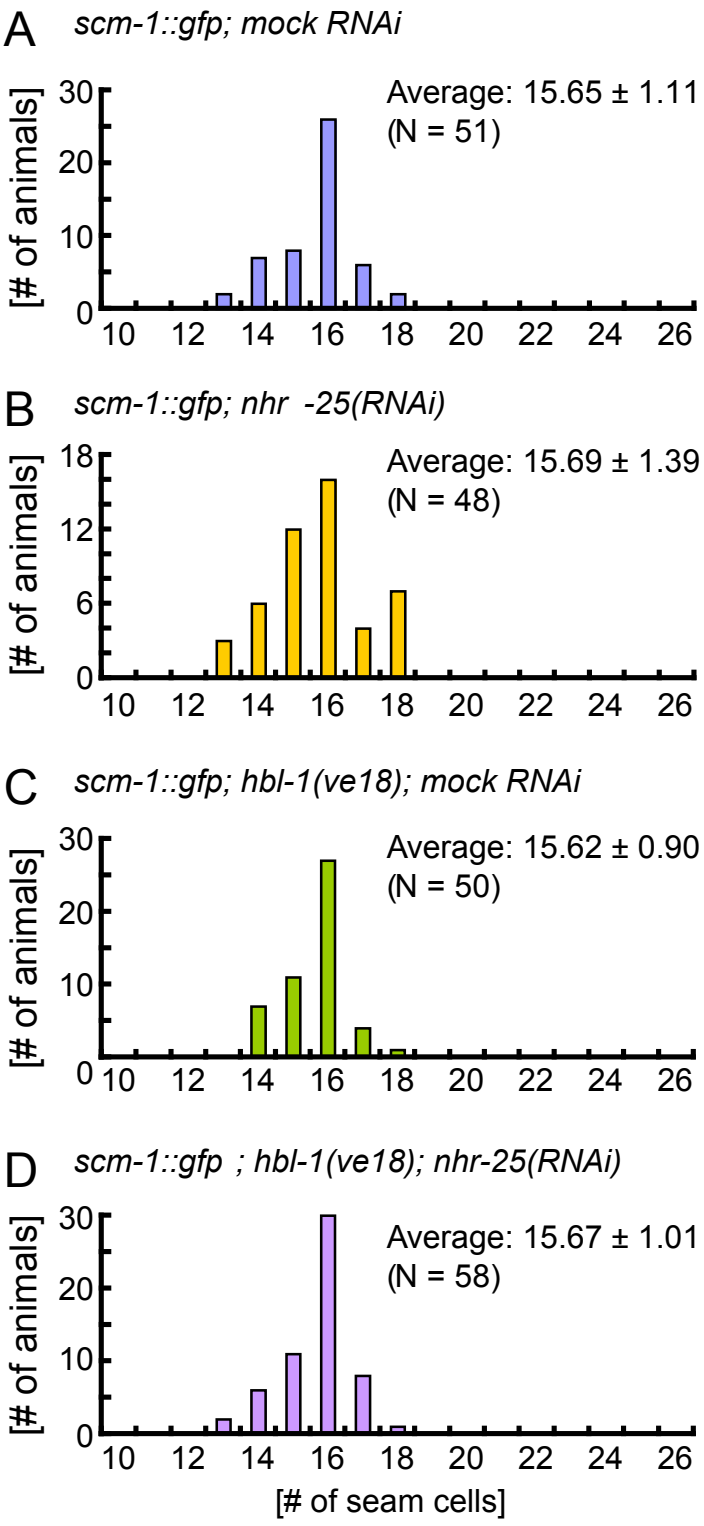

Figure S11
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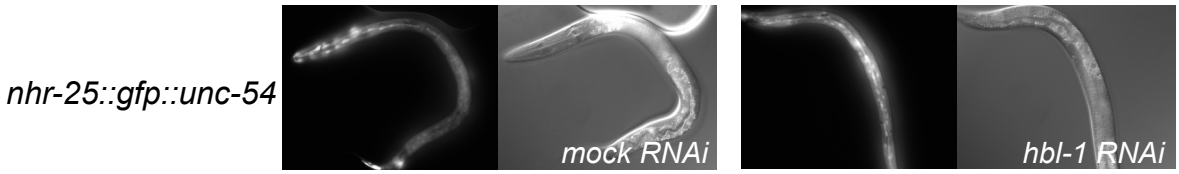

Supplementary Table S1: Genes for the RNAi screen in this study

"Plate" and "well" indicate positions of RNAi clones in the full genome-wide Ahringer's RNAi library that is commercially available from Geneservice. Genes that affected *apl-1* expression in seam cells are marked by yellow.

Gene	Plate	well	
ZC123.3	1	E4	
F54A5.1	1	G4	
C45E1.1	2	G7	
D1007.1	5	A5	
C04F1.3	7	F3	
F56H1.2	7	F5	
C32F10.6	7	G11	
C27A12.5	8	D4	
F26B1.7	9	B10	
T23H2.1	9	D8	
K10D3.3	11	A11	
C01H6.5	11	C6	
F55D12.3	12	F6	
F55D12.4	12	F7	
ZK265.4	13	C6	
F52B5.4	13	D8	
D1081.2	13	G1	
K02B12.1	13	G8	
F43G9.11	14	B5	
W10D5.1	15	B3	
C17E4.6	15	F8	
C36F7.1	16	A1	
Y67A6A.2	16	G1	
K10C3.6	16	G7	
T23H4.2	16	G9	
F52F12.6	16	H4	
C25A1.2	17	D8	
C25A1.11	17	E5	
ZC247.3	17	F5	lin-11
R13H8.1	18	G12	
C27C7.3	19	H9	
ZK1025.6	20	A6	
ZK1025.9	20	A9	
Y53C10A.3	21	A8	
Y53C10A.12	21	B5	
F41D3.1	21	E9	
F41D3.3	21	E11	
C47F8.2	21	G9	
C47F8.8	21	H3	
T09E11.2	21	H5	
T15D6.6	22	A8	
E03H4.6	22	B8	
E03H4.13	22	C2	
C54C8.1	22	C3	
Y26D4A.1	23	E8	
Y71A12C.1	25	C1	
ZK909.4	26	G1	
T27A1.6	32	B7	
ZC204.2	34	H4	
F45C12.2	35	B9	
F45C12.3	35	B10	
F45C12.15	35	C10	
F42G2.6	37	C7	
K12H6.1	38	C6	
K08A2.b	38	E8	
F19B10.9	40	F5	
C17A2.1	41	B12	

C17A2.8	41	C7
C49D10.2	41	C9
C49D10.6	41	D1
C49D10.9	41	D4
K10G6.1	41	E11
B0286.5	42	C4
ZK177.10	44	H12
F10C1.5	45	E9
F40H3.4	46	D12
K06A1.1	47	A9
K06A1.4	47	A12
F31E8.3	48	A6
F21D12.1	49	C12
F18A1.2	50	A6
F49E12.6	51	F2
F28C6.1	52	B2
F28C6.2	52	B3
C34C6.8	52	D10
T24H10.1	53	C6
T23G7.1	53	D7
E02H1.7	54	E6
Y53C12B.5a	54	H5
Y53C12C.1	55	A8
F26C11.2	55	C6
R53.3	55	D4
F27E5.2	55	G2
C07E3.5	56	B6
C07E3.6	56	B7
R06F6.6	57	B3
F40F8.5	57	G2
C47G2.2	58	A2
Y57A10B.4	60	B9
Y38E10A.f	60	G9
W01D2.2	64	C9
H10E21.3	66	A4
C29F9.13	66	C4
E02H9.8	68	D10
C48D5.1	69	C8
F34D10.5	69	E9
C28A5.4	71	A6
C38D4.6	71	H2
F26A1.2	71	H7
F21H11.3	72	G7
C34E10.7	73	A9
W03A3.1	74	C2
F40H6.4	74	H1
C16A3.7	75	F5
F31E3.1	76	G4
ZK418.1	77	A1
B0280.8	77	B5
H14A12.4	78	A1
C07H6.7	78	A11
R13A5.5	78	B3
C08C3.1	78	E3
C08C3.3	78	E5
ZK652.5	78	E12
C30A5.7	79	E4
C13G5.1	80	D11
T16H12.4	83	G6
ZK1128.4	83	H3
T07C4.2	84	C1
T07C4.6	84	C5
F43D9.5	84	E10
M142.4	85	C5

Y66A7A.8	86	B11
C24H11.3	86	D4
Y75B8A.1	86	G12
Y75B8A.2	86	H1
Y111B2D.e	87	E12
F53A2.4	88	E6
C37G2.5	89	E1
C05G6.1	93	A3
F55A8.1	94	D10
F52C12.5	94	E4
C37F5.1	94	G2
C09G12.1	95	D7
F28F9.1	95	H2
F36A4.14	96	H2
R11E3.5	98	A5
T28H11.4	98	D11
F26D12.b	99	F10
C45E5.6	99	H12
C17H12.9	101	D11
ZK381.3	101	G9
F33D4.1	103	E7
C26B2.3	104	D6
C26B2.4	104	D7
T09A12.4	104	G3
C09G9.7	106	C2
ZC410.1	106	E9
H22D14.1	107	B3
F14A5.1	107	B4
K01H12.3	108	B11
C28D4.1	108	C1
H27C11.1	108	C6
F32B6.1	108	F3
C04G2.7	109	C2
F01D4.6	110	C3
C08F8.8	111	H4
F54D1.4	112	B2
C29E6.5	113	D9
ZK829.5	113	E9
B0564.10	116	A12
Y62E10B.b	116	F1
F13G11.1	118	D2
Y51H4A.o	119	H12
Y51H4A.p	120	A1
Y116A8C.18	120	D7
C49C3.5	121	B6
Y41D4B_930.a	122	C10
Y41D4B_930.c	122	C12
Y59E9_120.a	123	A5
Y67D8A_386.b	123	D3
Y69A2A_7278.l	123	F12
R02C2.4	125	C12
F33E11.1	125	D5
ZK6.1	125	E5
ZK6.2	125	E6
ZK6.4	125	E8
ZK6.5	125	E9
M02H5.c	125	G4
M02H5.d	125	G5
T01G6.4	125	G6
M02H5.e	125	G12
T01G6.6	125	H2
T01G6.7	125	H3
T01G6.8	125	H4
R11G11.1	125	H7

R11G11.2	125	H8
R11G11.12	126	A6
C14C6.4	126	B1
ZK488.1	126	C5
ZK488.2	126	C6
ZK488.4	126	C7
F48G7.3	126	D4
F31F4.12	126	F2
R13D11.8	127	A5
C38C3.9	127	H10
F16B4.9	128	B6
Y46H3d.b	128	B11
ZK697.2	128	E3
Y5H2B.h	129	E2
F44C8.2	129	E5
F44C8.3	129	E6
F44C8.4	129	E7
F44C8.5	129	E8
F44C8.6	129	E9
F44C8.9	129	E12
F44C8.10	129	F1
F44C8.11	129	F2
F41B5.9	129	F11
T27B7.1	129	G4
T27B7.2	129	G5
T27B7.3	129	G6
T27B7.4	129	G7
T27B7.5	129	G8
F15E11.7	129	H5
T24A6.8	133	F10
T24A6.11	133	G1
T27C4.4	134	B7
F47C10.1	134	D2
F47C10.3	134	D4
F47C10.4	134	D5
F47C10.7	134	D7
C17E7.5	134	E1
C17E7.6	134	E2
C17E7.7	134	E3
T20C7.a	134	E8
T05B4.2	135	C2
R02D1.1	135	F4
T28F12.2a	136	A9
K03B4.3	136	B11
T19H12.8	136	F11
C10G8.7	137	G9
T09D3.4	137	H2
C24G6.4	138	B9
F44E7.8	138	G8
F17A9.6	139	D10
F44C4.2	140	D12
K11G9.4	140	F1
C33G8.8	141	C11
C33G8.9	141	C12
C33G8.12	141	D3
VC5.5	141	E6
C03G6.8	142	C8
C03G6.10	142	C10
C03G6.12	142	C12
C54F6.9	142	H11
F19F10.1	143	A5
F19F10.5	143	A9
C12D5.2	143	D7
C12D5.8	143	E1

D1014.9	144	D12	
T19A5.4	146	C9	
T19A5.5	146	C10	
F59E11.8	146	D6	
F59E11.10	146	D7	nhr-195
F59E11.11	146	D8	
C25E10.1	146	E11	
F44A2.4	147	C7	
F58E6.10	148	D3	
F57A8.5	149	A8	
Y22F5A.1	149	E12	
F29F11.5	150	E9	
T19B10.11	151	G3	
H12C20.3	152	E7	
W05E10.3	152	G5	
R07B7.13	153	E10	
R07B7.14	153	E11	
R07B7.15	153	E12	
C13C4.1	153	F2	
C13C4.2	153	F3	
C34D1.1	156	B1	
C34D1.2	156	B2	
C26E1.3	156	B9	
C50B6.8	156	C5	
F55B12.1	157	D8	
F09F3.10	157	E12	
R10D12.2	157	G11	
F58E10.2	158	A4	
F58E10.5	158	A7	
ZC376.4	158	D4	
F56A12.1	158	H6	
C15H11.8	159	A3	
ZK1037.4	161	C9	
ZK1037.5	161	C10	
R08H2.9	161	E9	
C06B8.1	161	H4	
K06B4.1	162	C9	
K06B4.5	162	D1	
K06B4.6	162	D2	
K06B4.7	162	D3	
K06B4.8	162	D4	
K06B4.10	162	D6	
F57A10.5	162	F9	
T26E4.8	162	G5	
F54B8.2	162	H2	
T06C12.7	163	A12	
F35E8.12	163	C7	
F36G9.12	163	E4	
C06C6.5	163	E12	
F14H3.11	163	H5	
T13F3.2	164	E3	
T13F3.3	164	E4	
F57G8.6	164	G3	
F36D3.2	165	C7	
Y32B12B.6	165	D9	
T03E6.3	165	D12	
Y70C5C.6	165	H1	
F09C6.8	166	C6	
F09C6.9	166	C7	
Y102A5C.18	166	D11	
ZK218.6	166	H8	
Y17D7B.1	170	A8	
C54E10.5	170	B5	
Y17D7A.1	170	B7	

Y17D7A.2	170	B8	
Y80D3A.g	170	C5	
Y39B6B.d	170	E4	
Y116F11A.zz4	172	B5	
F38A6.1	173	H1	
F38A6.3	173	H3	
R04A9.5	176	A12	
ZK1193.5	176	B5	
K06A9.2	178	A8	
ZK380.1	178	B6	
C36C9.2	178	C2	
T26C11.1	178	E8	
T26C11.5	178	E12	
T26C11.6	178	F1	
F56E3.4	180	F4	
K09C4.5	180	G7	
C12D12.5	181	B9	
C02F12.5	181	E7	
T14G12.4	181	F4	
ZC64.3	181	H1	
ZC64.4	181	H2	
F48D6.1	182	D3	
C52B9.2	182	D8	
C07A12.1	182	H5	
C07A12.3	182	H7	
F16H11.5	183	B4	
C39E6.4	183	C8	
C42D8.4	183	G9	
W01C8.2	184	F7	
T13C5.4	185	E5	
F22A3.1	186	A6	
F22A3.5	186	A10	
C25B8.6	186	B11	
C56E10.1	186	B12	
C56E10.4	186	C3	
F46C8.5	187	G4	
T01B10.4	189	D11	
R07B1.1	191	E1	
F44A6.2	192	B4	
C33D3.1	192	E6	elt-2
F14F3.1	192	E9	
F11A1.3	192	G6	
F58A3.1	193	C11	
ZK455.6	193	H7	
H05G16.1	194	C1	
C40H5.5	194	F1	
F54F7.1	194	F8	
T08D10.1	194	H8	
C49F5.4	195	A2	
F17A2.5	195	E8	
T24D3.1	195	G7	
T18D3.2	195	G9	
T18D3.7	195	H2	
C02B4.2	196	B3	
T07C5.2	196	B6	
T07C5.3	196	B7	
T07C5.4	196	B8	
T07C5.5	196	B9	
F16H9.2	196	B11	
F11C1.6	196	E10	nhr-25
K03A11.3	196	F4	
C29F7.5	197	C10	
K11E4.5	197	H7	
K02B9.4	198	B7	

F16B12.8	198	D12
F28H6.2	198	E2
C37E2.5	198	F3
K04C1.3	198	F9
H01A20.1	199	B8
C27C12.6	199	F5
C18B12.3	199	H10
C33A11.4	200	D8
T27A8.2	201	C7
R09G11.2	203	D9